GENETIC CONTROL OF CELL-MEDIATED RESPONSIVENESS TO AN AKR TUMOR-ASSOCIATED ANTIGEN

Mapping of the Locus Involved to the *I* Region of the *H-2* Complex*

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The observation by Lilly et al. (1) that high leukemia mouse strains (AKR, C58, RF, and C3H/Fg), as well as the two strains utilized by Gross to demonstrate a filterable virus agent from AKR mice, C3H/Bi, and C57BR, are all of the $H-2^k$ haplotype, suggested that a relationship existed between susceptibility to leukemogenesis and the $H-2^k$ haplotype. Further study of Gross virus-induced leukemia incidence in the cross C3H ($H-2^k$) × C57BL ($H-2^b$) indicated quite clearly that a gene closely linked to the $H-2$ complex, $Rgv-1^1$ (resistance to Gross-virus-1), was one of two independently segregating loci sharing in the control of susceptibility to leukemogenesis (2).

The importance of $Rgv-1$ in affecting susceptibility to Gross virus-induced leukemia was subsequently corroborated in studies with congenic strains of mice which differed from each other only with regard to the $H-2$ chromosomal segment. Thus, mice of the congenic C3H ($H-2^k$) and C3H.SW ($H-2^b$) strains differed markedly in their susceptibility to Gross virus, being highly susceptible and highly resistant, respectively (3). Several other lines of evidence also indicated an effect of $H-2$ on susceptibility to viral leukemia in mice. For example, Tennant and Snell (4) studying leukemogenesis by the BALB-Tennant leukemia virus (BT/L) virus noted considerably higher levels of resistance in C57BL/10 ($H-2^b$) than in congenic B10.BR ($H-2^k$) mice.

The first indication of the mechanism of action of $Rgv-1$ was obtained while studying $H-2$ effects on Friend virus-induced erythroleukemia (5). Susceptible mice required a tenfold lower Friend virus (FV) dose for splenomegaly induction

¹ *Abbreviations used in this paper:* BT/L, BALB-Tennant leukemia virus; CML, cell-mediated lympholysis; F_1, first filial generation; FCS, fetal calf serum; FV, Friend virus; $H-2$, murine major histocompatibility complex; Ir, immune response; LCM, lymphocytic choriomeningitis virus; LNC, lymph node cells; MEM, Eagle's minimum essential medium; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; $Rgv-1$, resistance to Gross virus-1; TSTA, tumor-specific transplantation antigen.

and were much less prone to recovery from splenomegaly than resistant mice, indicating that the H-2 difference of the hosts appeared to significantly alter the course rather than the onset of the disease.

Studies with H-2 recombinant mice indicated that Rgv-1 maps within the K end of the H-2 complex (3), comprising the K and I regions. The clear demonstration of several genes that regulate the level of immunological responses (Ir genes) to a variety of natural and synthetic antigens mapping within the I region of the H-2 complex (6, 7); the close or identical mapping of Rgv-1 with the I region; and the indication from FV disease studies that the H-2 effect may influence a late event in the disease, namely recovery from splenomegaly (5), suggested a possible identity between Rgv-1 and Ir gene(s) (7, 8). Thus, H-2-linked resistance to virus-induced leukemogenesis may result from a stronger immunological response to a given virus-induced antigen.

One indication that this hypothesis might be corrrect is the finding of Aoki et al. (9) in studies with Gross virus that a significant number of mice homozygous or heterozygous for the H-2^b haplotype showed detectable levels of anti-Gross virus antibodies, but no H-2^k homozygotes produced measurable antibodies. In experiments by Sato et al. (10), several leukemias derived from BALB/c mice were rejected by hybrids of BALB/c with other inbred strains, contrary to the usual rules of transplantation. Genetic studies on a series of hybrids established that responsiveness to these tumors was linked to the K end of H-2, the same location as Rgv-1 and the majority of H-2-linked Ir genes. Furthermore, animals resistant to the tumor had higher titers of antibody to Gross-specific antigens than susceptible animals.

In addition, genetic control of susceptibility to infection with lymphocytic choriomeningitis virus (LCM) has been demonstrated by Oldstone et al. (11). Susceptibility to LCM, a dominant trait, is associated with the H-2^q haplotype, whereas resistance is found with the H-2^k genotype. Because the pathogenic consequences of LCM infection result from the immune response, it has been proposed that Ir genes may be responsible for H-2-linked susceptibility or resistance to LCM infection (12).

The studies presented here were undertaken to test the hypothesis that H-2-linked genetic control of resistance to leukemogenesis results from a heightened immune response in the resistant animal. In brief, AKR mice were crossed with animals of various H-2 congenic strains on the C3H and C57BL/10 genetic backgrounds, and the hybrid mice injected with tumor cells from an established, tissue culture-adapted AKR thymoma. The resulting cell-mediated immune response of such hybrids was studied. The results demonstrate that the ability to generate a primary or secondary cell-mediated response to an AKR tumor cell antigenic determinant is under H-2 linked control and the locus determining cell-mediated lympholysis (CML) responsiveness maps in the I-J subregion.

Materials and Methods

Cell Lines Used. BW5147, an AKR-derived cell line adapted to culture in 1972 from a spontaneous cell tumor originating at The Jackson Laboratory, Bar Harbor, Maine in 1954 (13), and EL-4, a C57BL/6-derived cell line adapted in 1970 from acute (benzo [α-] pyrene-induced leukemia; reference 13), were obtained from the Salk Institute. AKR mouse embryo fibroblast

TABLE I

Haplotype Origin of Region Carried by Strains Used in Present Work

Strain	H-2 haplotype	Haplotype origin of region H-2								
		K	A	B	J	E	C	S	G	D
AKR	H-2^k	k	k	k	k	k	k	k	k	k
C3H.SW	H-2^b	b	b	b	b	b	b	b	b	b
C3H.Q	H-2^q	q	q	q	q	q	q	q	q	q
C3H	H-2^{k*}	k	k	k	k	k	k	k	k	k
CKB	H-2^{k*}	k	k	k	k	k	k	k	k	k
B10.G	H-2^q	q	q	q	q	q	q	q	q	q
B10.S	H-2^s	s	s	s	s	s	s	s	s	s
C57BL/10	H-2^b	b	b	b	b	b	b	b	b	b
B10.BR	H-2^k	k	k	k	k	k	‡k	k	k	k
B10.A	H-2^a	k	k	k	k	k	| d	d	d	d
B10.A (1R)	H-2^{i1}	k	k	k	k	k	d	d	d |	b
B10.A (2R)	H-2^{i2}	k	k	k	k	k	d	d	d |	b
B10.A (3R)	H-2^{i3}	b	b	b	b |	k	d	d	d	d
B10.A (4R)	H-2^{i4}	k	k |	b	b	b	b	b	b	b
B10.A (5R)	H-2^{i5}	b	b	b |	k	k	d	d	d	d

* CKB and C3H mice differ in their allotype.

‡ Vertical bar indicates crossover position.

were isolated in our laboratory from a 14-day pregnant AKR mouse and established as a 3T3 cell line by the methodology described by Paul (14).

Studies by Shröder, personal communication, have shown that the BW5147 cell line had 43, sometimes 44 or 86, chromosomes, and most of them could be identified as part of the normal mouse complement. The majority of the cells have identical karyotypes, although minor variation or tetraploidy is found in a few. In general, one pair of each chromosome is represented, except for chromosomes 3, 18, and X. Only one normal chromosome number 3 is found, the other being part of a translocation, and both chromosomes 18 are present in an isochromosome 18. The cell line has only one X chromosome. By banding analysis, the line appears to lack a Y chromosome and thus is phenotypically female. A few marker chromosomes of unidentifiable origin are also present. Detailed studies of the translocation involving one of the chromosomes number 3 indicated that the other chromosome is number 17 with a terminal deletion. Thus, the BW5147 cell line is trisomic for most of chromosome 17.

BW5147 is θ-AKR$^+$ (13), TL^- (15), H-2^{k+} (13), and PHA sensitive (13). EL-4 cells are G^+, H-2^{b+}, θ-C3H$^+$, TL^-, Ly-1.2$^+$, surface Ig$^-$, cortisone resistant, and PHA sensitive (13).

Mice. AKR/J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. All other mouse strains were bred and maintained in our own colonies.

H-2 Typing of Backcross Progeny. National Institutes of Health contract antiserum D-17 ([D1.C × AKR.M]F$_1$ anti-DBA.1 [anti-H-$2K^q$]) and an (A.TL × B10.A[5R]F$_1$ anti-B10.A [anti-H-$2K^k$]) antiserum raised in our laboratory were used to H-2 type (AKR × B10.G)F$_1$ × AKR progeny by the hemagglutination procedure (16).

Preparation of Effector Cells. Animals were killed by cervical dislocation and their spleen and mesenteric lymph nodes removed by sterile technique in phosphate-buffered saline (PBS) + 5% fetal calf serum (FCS), and single cell suspensions prepared with gentle teasing and pipetting. Peritoneal exudate cells were obtained by flushing the peritoneal cavity with sterile PBS.

Preparation of Target Cells. BW5147, EL-4, or AKR mouse embryo fibroblast cells (viability greater than 90%) were incubated at a concentration of 10 × 10^6/ml in Dulbecco's minimal essential medium (MEM)-10% FCS with 1 mCi/ml of ^{51}Cr for 1 h at 37°C (in a CO$_2$ incubator). The reaction was terminated by adding cold PBS + 5% FCS. Cells were spun and washed three times or until the amount of ^{51}Cr remaining in the supernatant fluid was <5% of the amount remaining in the cell pellet.
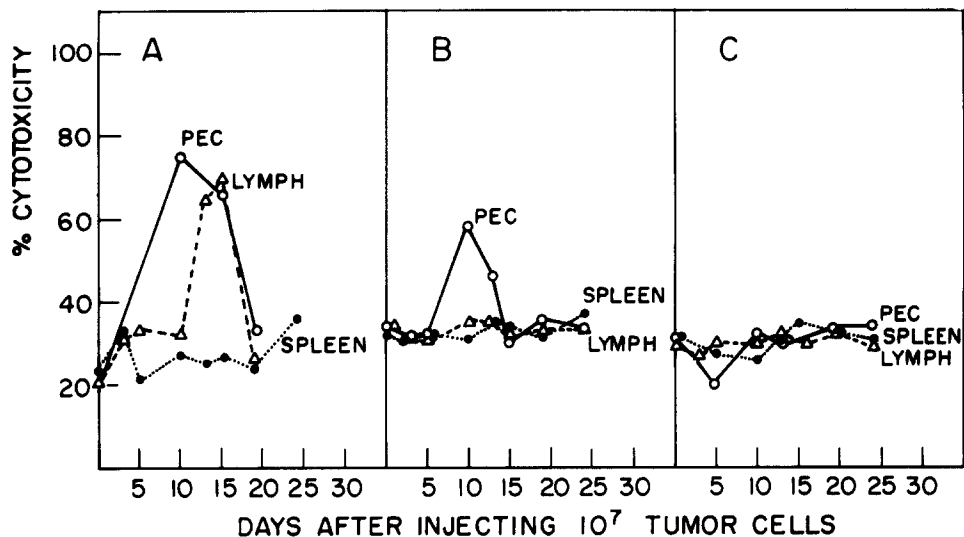
FIG. 1.   10 × 10⁶ AKR tumor cells were inoculated into (AKR × B10.BR)F₁ hybrids at various intervals. At the conclusion of the experiment all animals were sacrificed and cell suspensions shown were tested in a 12-h cytotoxic test against ⁵¹Cr-labeled AKR tumor cells as described in Materials and Methods. The assay was performed with three different effector to target cell ratios: (A) 600:1; (B) 60:1, and (C) 15:1.

Assay of CML.   Effector cells were incubated with target cells in a total volume of 0.3 ml of Dulbecco's MEM + 10% FCS at 37°C in a CO_2 incubator for 12–16 h in a microwell dish (IS-MRC-96 from Flow Laboratories, Inc., Rockville, Md.). The microwell dish was then centrifuged at 1,000 rpm for 10 min and 0.1 ml of the supernate was removed and counted, in a Searle Analytic (Nuclear Chicago) gamma counter (1185 series; Searle Analytic Inc., Des Plaines, Ill.). Unless otherwise indicated, the results are expressed as percent specific lysis, which is calculated by the formula:

$$\% \text{ specific lysis} = \frac{\text{experimental } ^{51}\text{Cr release-spontaneous } ^{51}\text{Cr release}}{\text{maximum } ^{51}\text{Cr release-spontaneous } ^{51}\text{Cr release}} \times 100$$

where maximum release is defined by the ⁵¹Cr cpm obtained after freezing and thawing the labeled target cells six times; spontaneous release is leakage of ⁵¹Cr cpm from target cells unexposed to effector cells, i.e., target cells incubated alone in media for the duration of the experiment (spontaneous release usually was 20 ± 5%).

Results

Kinetics and Specificity of the Cell-Mediated Response to AKR Tumor Cells.   F₁ mice produced by mating AKR/J with various C3H and B10 *H-2* cogenic strains were inoculated with 10⁷ AKR tumor cells (BW5147) at various intervals, sacrificed at the conclusion of the experiment, and cell suspensions of spleen, mesenteric lymph node (LNC), and peritoneal exudate (PEC) were prepared to test for the presence of cytotoxic killer cells on ⁵¹Cr-labeled AKR tumor cells.

Results shown in Fig. 1 indicate that (a) maximum cytotoxicity is obtained from LNC and PEC 10 to 15 days after injection of BW5147 tumor cells, and (b) no cytotoxic or killer cells are apparent in any of the spleen cell populations tested. Subsequent experiments, designed to test the target specificity of the response, indicated that PEC effectors are not always specific for AKR tumor
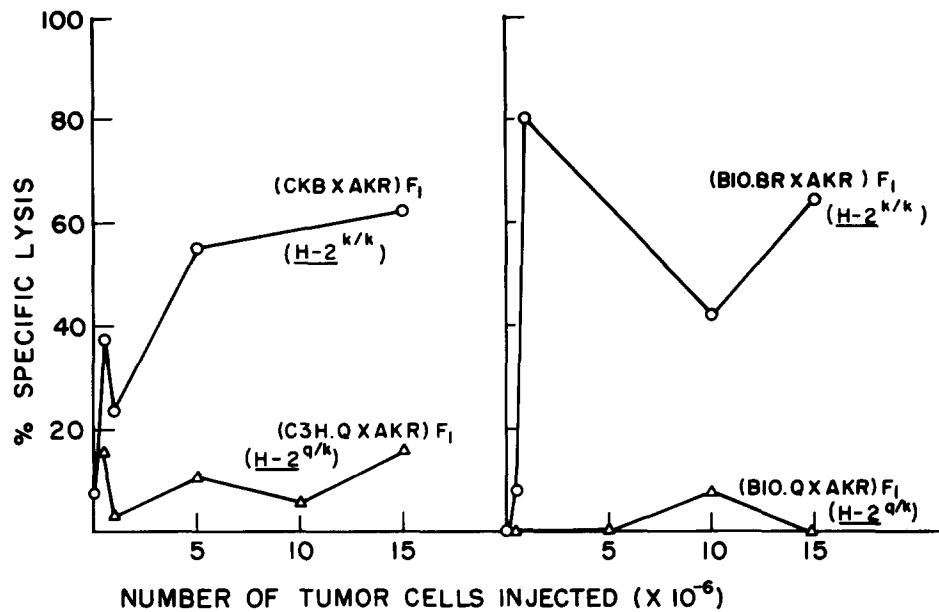
Fig. 2. Cell-mediated responses of 12 × 10⁶ mesenteric lymph node cells from animals immunized 10 days earlier with the indicated number of tumor cells. 34 × 10³ ⁵¹Cr-labeled AKR tumor cells were used as targets for each assay. The lysis represents the percent of label released after 16 h of incubation with effector cells.

cells, but also lyse other cell types, such as EL-4 tumor cells and normal AKR embryo fibroblasts. The target specificity of LNC was always strictly restricted to the AKR tumor cells used in the in vivo challenge. All subsequent work was carried out with the more selective LNC effector population.

Genetic Regulation of CML Responsiveness. A comparison made of the cell-mediated immune responsiveness to BW5147 of different F_1 hybrid cells revealed apparent genetic regulation. Fig. 2 shows the response of four such hybrids; (CKB × AKR)F_1 and (B10.BR × AKR)F_1 both respond vigorously and are homozygous at *H-2* (*H-2$^{k/k}$*). The difference in their non-*H-2* genetic composition does not affect the response. On the other hand, (C3H.Q × AKR)F_1 and (B10.G × AKR)F_1, both identical in their *H-2* genotypes, namely *H-2$^{q/k}$* (but, again, different in their non-*H-2* genetic make up), fail to respond. These results indicate that the *H-2$^{q/k}$* genotype results in nonresponsiveness, whereas the *H-2$^{k/k}$* genotype results in responsiveness, independent of the non-*H-2* genetic background of the F_1 strains used.

The observed differences in responsiveness could be attributed to a genetically controlled difference in immune responsiveness. Another explanation, however, involves the possibility of differential growth of the injected tumor cells in different F_1 hybrids. The more rapid growth in one F_1 strain might serve as a stronger antigenic stimulus, or alternatively, rapid invasiveness by the tumor cells might lead to greater impairment of the immunological apparatus and capacity to respond. To study this possibility, irradiated (3,000 rads) BW5147 cells were inoculated into F_1 hybrid mice of the *H-2$^{k/k}$* and *H-2$^{q/k}$* genotypes. When cells are exposed to 3,000 rads, they remain antigenically intact, and
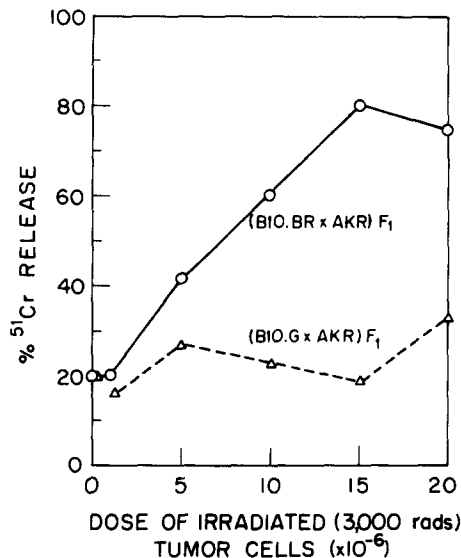
Fig. 3. Conditions were as described for Fig. 2, except that tumor cells inoculated were exposed to 3,000 rads in a Cessium irradiator before injection.

viable by the criterion of exclusion of trypan blue, but are unable to multiply and grow. As shown in Fig. 3, when irradiated cells were inoculated into these same hybrids, H-$2^{k/k}$ animals responded and H-$2^{q/k}$ animals failed to respond, indicating that the results previously obtained could not be ascribed to differential growth rates of the AKR cells in the different hybrids.

H-$2^{q/k}$ animals remained unresponsive in a secondary CML response (irradiated tumor cells were injected, followed 25 days later by inoculation of live cells), whereas H-$2^{k/k}$ F_1 mice mounted a vigorous secondary response (data not shown).

Formal proof of linkage between cell-mediated responsiveness and H-2 was obtained by carrying out a backcross segregation analysis. (B10.G × AKR)F_1 mice (H-$2^{q/k}$) were backcrossed to AKR (H-$2^{k/k}$) mice. The predicted outcome of such a backcross is 50% H-$2^{k/k}$ animals, all of which should be responsive, and 50% H-$2^{q/k}$ mice, all of which should be unresponsive. Out of 20 such animals tested (Fig. 4), 9 were H-$2^{q/k}$ and completely unresponsive and 11 were H-$2^{k/k}$, 8 of which demonstrated a normal cytotoxic response. These results are in good agreement with the predicted outcome for H-2 linkage of CML responsiveness. The reason why 3 out of 11 H-$2^{k/k}$ animals failed to respond is unclear. Experimental error at some point might have occurred, but it is equally as likely that biological variation, or additional genetic interactions, may be responsible for the three unresponsive H-$2^{k/k}$.

Strain Distribution Survey of F_1 Nonresponsive Trait. The above results illustrate that homozygous H-$2^{k/k}$ animals are able to respond, whereas heterozygous H-$2^{q/k}$ animals are not, a finding which suggests that responsiveness is regulated either by (*a*) a recessive trait or (*b*) a dominant suppressor gene(s) which causes nonresponsiveness. This is an unusual finding in that most H-2-linked *Ir* genes defined to date have shown dominant responsiveness (6);
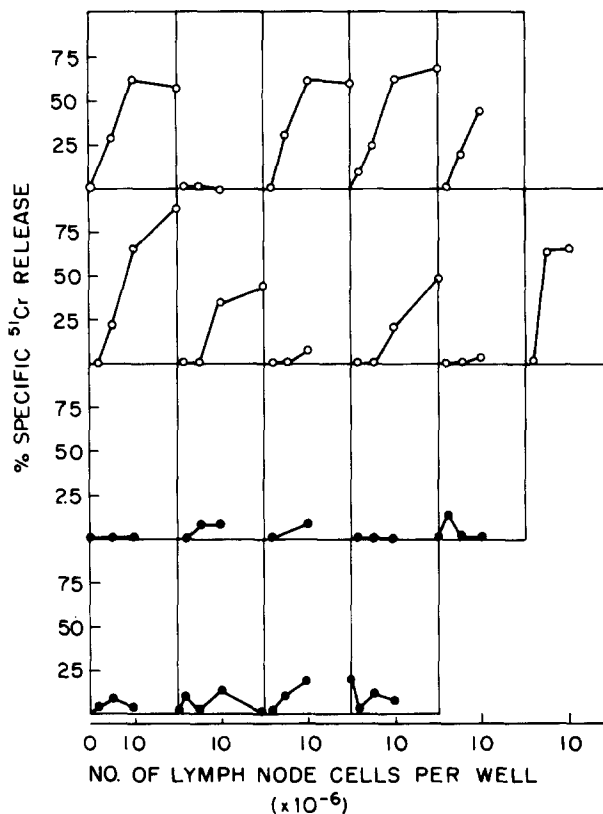
Fig. 4. Cell-mediated responses of (B10.G × AKR)F$_1$ × AKR mice inoculated 14 days earlier with 5 × 10^6 AKR tumor cells as a function of increasing numbers of lymph node cells (shown) to a constant number of ^{51}Cr-labeled AKR target cells (90,000). Mice were H-2 typed by hemagglutination assay. 11 mice were H-2$^{k/k}$ (O———O) and 9 mice were H-2$^{q/k}$ (●———●). Incubation time of CML assay was 14 h.

however, several workers have recently demonstrated the existence of H-2 linked, dominant immune suppression gene(s) (17, 18).

It is, therefore, important to determine whether any H-2 heterozygous animal is capable of a cell-mediated cytotoxic response to AKR tumor cells, or whether only H-2$^{k/k}$ homozygous mice are able to respond. Should some heterozygous mice be found to respond, the notion of suppression, in contrast to recessive responsiveness, would be supported. Fig. 5 shows the results obtained when other H-2 heterozygous animals were tested. H-2$^{b/k}$ F$_1$ mice ([C.SW × AKR]F$_1$) failed to respond (as did H-2$^{q/k}$ F$_1$ [B10.G × AKR]F$_1$ or [C3H.Q × AKR]F$_1$), but H-2$^{s/k}$ F$_1$ ([B10.S × AKR]F$_1$) mice generated a cell-mediated cytotoxic response comparable to that of homozygous H-2$^{k/k}$ mice ([B10.BR × AKR]F$_1$ or [C3H × AKR]F$_1$). The fact that H-2$^{s/k}$ F$_1$ respond suggests that responsiveness is not a recessive trait. However, it could be argued that the H-2^s and H-2^k haplotypes share the same allele as the gene(s) responsible for generation of a cell-mediated cytotoxic response, and an H-2$^{s/k}$ F$_1$ animal responds because it is homozygous for the recessive allele at this locus.
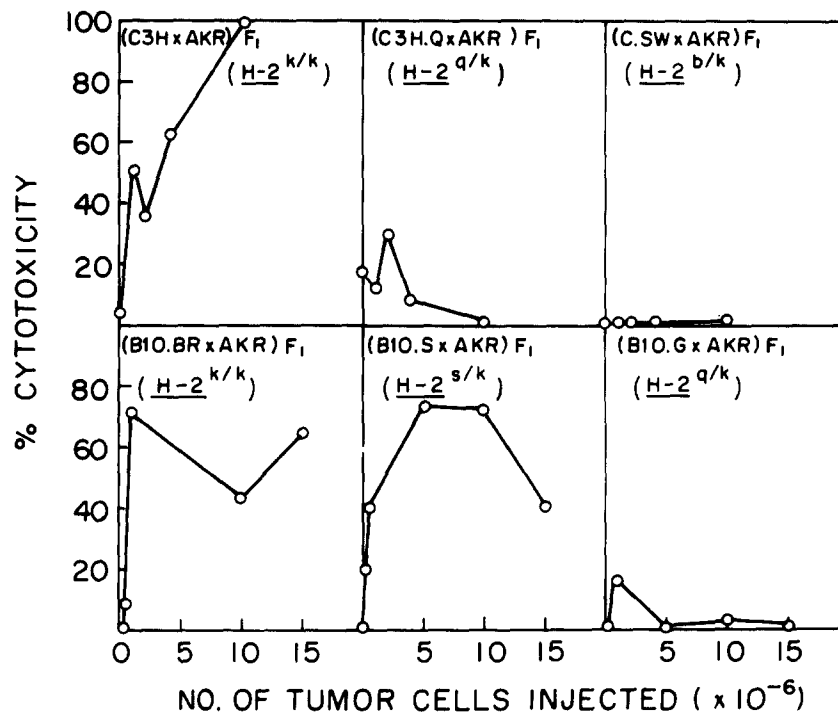
FIG. 5.   Cell-mediated cytotoxic response of 10^7 lymph node cells to 50×10^3 ^{51}Cr-labeled AKR tumor cells. Animals were injected 10 days earlier with the indicated number of tumor cells. Effector and target cells were incubated for a total of 12 h.

Mapping of CML Responsiveness to the I-J Subregion.   As seen in Fig. 5, (C.SW × AKR)F$_1$ mice (H-$2^{b/k}$) do not make a cytotoxic response to the AKR tumor cells, at least under the experimental conditions used. Further tests with H-$2^{b/k}$ mice have shown that nonresponsiveness in this hybrid is not absolute. H-$2^{k/b}$ mice can respond if injected with a sufficiently large dose of tumor cells and if a sufficiently high ratio of lymph node effector to target AKR cells is tested. The finding that H-$2^{k/b}$ mice are low responders in terms of cell-mediated immunity to AKR tumor cells is a fortunate one, because it allows more precise genetic mapping within the H-2 complex of the locus responsible for nonresponsiveness. A substantial number of available H-2 recombinant mice have been derived from a crossover between the H-2^b and H-2^a haplotypes. The H-2^a haplotype carries genes identical to the H-2^k genotype throughout most of the I subregions of the H-2 complex. Because the latter haplotype (H-2^k) carries the responder allele(s), available recombinants were examined for CML responsiveness. Fig. 6 shows the CML response of various H-2 recombinant F$_1$ mice to two doses of tumor cells. It can be seen that when 5×10^6 AKR tumor cells are injected, animals having only the H-2^k allele(s) in the I-J subregion respond, whereas those having the H-2^b allele(s) do not. However, at the higher dose of tumor inoculation (10^7 cells), animals having the I-J^b allele respond if high effector to target ratios are tested. These data tentatively map a locus regulating CML responsiveness to AKR tumor cells to the I-J subregion. In light of the fact that most presently known genes associated with
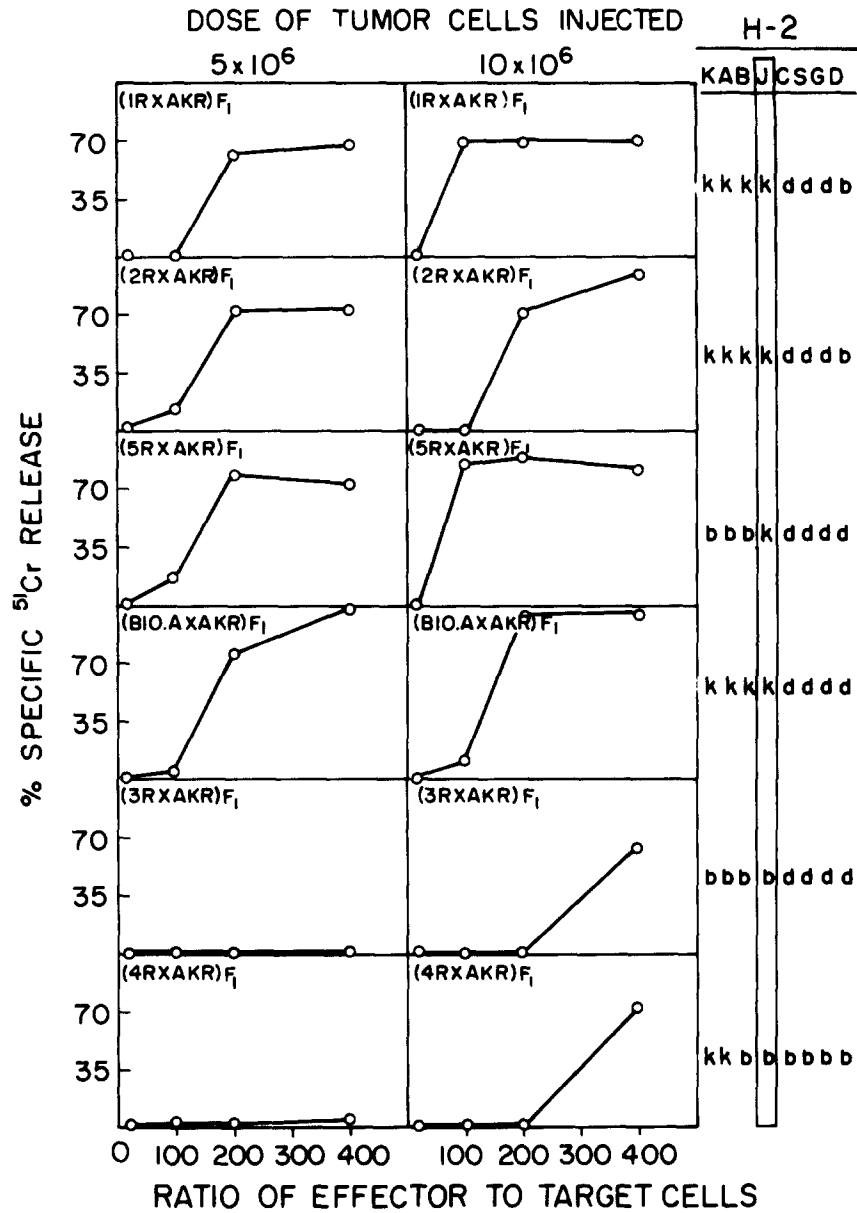
DOSE OF TUMOR CELLS INJECTED

H-2

FIG. 6. Cell-mediated responses of various recombinant mice bearing the indicated *H-2* haplotype to two different doses of tumor cells (numbers at top) injected 10 days earlier. In each test, various ratios of effector to target cells were used. Incubation time was 14 h.

the *I-J* subregion appear to code for antigenic determinants present on suppressor T cells (19) and soluble factors from suppressor T cells (20), such mapping provides further circumstantial evidence for considering the cell-mediated unresponsiveness to the tumor to result from the action of suppressor T lymphocytes.

Discussion

Data presented in this report, indicating that H-$2^{q/k}$ and H-$2^{b/k}$ hybrid mice fail to respond to an AKR thymoma-derived cell line, represent the first direct demonstration of an H-2 linked, cell-mediated immune response difference to a (presumed) tumor-specific transplantation antigen (TSTA). The resulting CML has been shown to be specific for the AKR tumor cells injected, and not effective against normal cells or unrelated tumor cells. It is, however, not clear whether the response is directed against a TSTA, a virus-associated antigen, or an antigenic change in the cell resulting from its maintenance in tissue culture. This finding may be of major significance because such gene(s) have long been postulated in explaining the association between leukemia susceptibility and H-2 in mice (7, 8). The definition of the I-J subregion is based on studies of Ia antigenic determinants present on suppressor T lymphocytes or their factors (19, 20). No Ir gene is yet assigned to this subregion. Although the mapping data included in this study can only be considered preliminary — it is based on only two experiments which included the key strain pair comparison, i.e. (B10.A[5R] × AKR)F$_1$ vs. (B10.A[3R] × AKR)F$_1$ — these data, if correct, assign an immune suppression gene to this subregion.

Initial studies indicate that H-$2^{k/k}$ homozygotes significantly outlive unresponsive H-$2^{b/k}$ heterozygous mice after secondary and tertiary challenge with AKR tumor cells, but no survival difference can be detected to the initial tumor challenge among the various hybrids. (It is important to note that most animals, including responsive F$_1$ hybrids, ultimately die from the growing tumor load, even though quantitative differences in survival times can be detected after more than one challenge.) This result is somewhat unexpected in view of the fact that AKR (H-2^b) mice show a markedly lower incidence of and longer survival with spontaneous Gross virus-induced leukemia than do AKR (H-2^k) mice (21). This may be because the experimental system used looks at a terminal stage of the disease, in which the tumor level has reached greater than 1×10^6 cells. It is possible that immune suppression genes operating at this late stage of the disease are considerably different from Ir genes operating earlier, during the initial onset of the disease. Nowinski et al. (22) have recently described genetically controlled immune responses which operate in the preleukemic animal, and defined such immunity as "natural immunity." Such genetic control is clearly distinct from the one described here.

It will be important to understand the types of lymphocytes involved in the cellular responses described in the present system, as well as the interactions among such cells. The present studies do not answer the question of whether a recessive trait coding for CML responsiveness, a dominant suppressor mechanism, or an alternate mechanism is operating in the present experimental system. Further direct tests to distinguish between these mechanisms are currently in progress.

The possibility that H-2 control of virus-induced leukemogenesis may, at least in some instances, result from the action of genes distinct from Ir genes should also be considered. Recent studies by Chesebro et al. (23) indicate that resistance to FV leukemogenesis is associated with the H-$2D$ locus of the H-2 complex. In a series of experiments designed to test directly the role of Ir genes

on FV disease, Chesebro et al. (24, 25) found that little correlation existed between H-2 genotype and ability to generate either a cell-mediated or humoral response. Both susceptible (H-2^k) and resistant (H-2^b) animals were capable of generating vigorous cell-mediated and humoral responses. In addition, H-2 control of BT/L (4), mammary tumor virus (26), and radiation leukemia virus (27) appear to be associated with the D rather than the K end of the complex. It is clear, however, that although alternative H-2-linked mechanisms of defense may exist, our understanding of all such mechanisms is of major concern in view of their possible role in the association between HLA and a variety of human neoplasms (28).

Summary

The role of H-2-linked genes in controlling resistance to murine leukemia viruses has been studied by measuring the cell-mediated immune response of F_1 hybrid mice (between AKR and various C3H and C57BL/10 derived, H-2 congenic strains) to an AKR tumor cell line, BW5147. The studies have shown that the ability to generate a primary or secondary cell-mediated response to an AKR tumor cell antigenic determinant is under H-2 linked control. The locus determining CML responsiveness maps in the I-J subregion. Nonresponsiveness is associated with the H-$2^{q/k}$ and H-$2^{b/k}$ hybrid genotypes, whereas responsiveness is associated with the H-$2^{k/k}$ homozygous genotype. Nonresponsiveness may result from (a) dominant suppression; (b) recessive responsiveness; or (c) an alternate mechanism not yet understood. This type of control may be one of several H-2-associated mechanisms of defense against virus-induced neoplasms.

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