A ROLE FOR ELEVATED H-2 ANTIGEN EXPRESSION IN RESISTANCE TO NEOPLASIA CAUSED BY RADIATION-INDUCED LEUKEMIA VIRUS

Enhancement of Effective Tumor Surveillance by Killer Lymphocytes*

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Resistance to radiation-leukemia virus-induced leukemia is mediated by gene(s) in the H-2D region of the major murine histocompatibility complex (1). The mechanisms by which gene(s) in this region affect leukemogenesis are hitherto unknown. However, previous observations (2) suggest a role in disease resistance for changes in H-2 expression occurring immediately after virus inoculation. For example, gene products (antigens) of the H-2D region show the most marked and prolonged changes in expression after virus inoculation. In addition, elevated H-2D antigen expression after virus inoculation occurs for thymocytes of resistant but not susceptible mice. Furthermore, there is an inverse relation between expression of H-2D and viral antigens. Viral antigen expression is greater in susceptible animals, whereas H-2D antigen expression is maximal for virus-infected resistant mice. Finally, H-2 antigens usually disappear from the surface of radiation-induced leukemia virus (RadLV)1 transformed cultures when overt leukemia develops. Thus, resistance to the disease is associated with increased H-2 antigenic expression, and the onset of leukemia is associated with disappearance of these antigens.

Several mechanisms by which changes in H-2 antigen expression may overtly influence the course of the disease have been previously suggested (2). Although the effect of increased H-2 expression on the other steps required for oncogenesis remains to be evaluated, the observations reported herein suggest that elevated H-2 antigen expression enhances the effectiveness of the host's immune responses to virus-infected cells and may function in resistance to leukemogenesis.

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‡ Abbreviations used in this paper: CMC, cell-mediated cytotoxicity; CMI, cell-mediated immunity; CML, cell-mediated lymphotoysis; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GAMIG, goat anti-mouse IgG; H-2, histocompatibility-2 complex; Ir, immune response; NMS, normal mouse serum; NP-40, Nonidet-P40 detergent; PBS, phosphate-buffered saline; RadLV, radiation-induced leukemia virus; SDS-PAGE, sodium dodecyl-sulfate-polyacrylamide-gel electrophoresis; TL, thymus-leukemia.
Materials and Methods

Mice. All animals used in the present study were bred either at Stanford University School of Medicine or at New York University Medical Center from animals derived from the Stanford colony, and generously provided by Dr. Hugh O. McDevitt.

Virus. Initial virus extract (RadLV) was derived from virus-induced lymphoid tumors of C57BL/Ka mice as previously described (3). This virus has been passaged independently for 1 yr. It is highly leukemogenic in vivo, exhibits the same host restrictions (H-2 control) as the original RadLV prepared from C57BL/Ka lymphoid tumors, and shows the same sodium dodecyl sulfate polyacrylamide gel electrophoresis polypeptide pattern (D. Meruelo, unpublished observations).

H-2 Antisera. H-2 alloantisera 056 ((B10.BR X A.SW) F1 anti-B10.S(7R) (anti-H-2Dk)), and 020 ((A.TH anti-A.SW) (anti-H-2Dk)) were generously provided by Dr. Hugh O. McDevitt. Antiserum D-30 ((B10.A X LP.RIII) F1, anti-B10.AKM) (anti-H-2Dq)) and D-11b ((A.SW X B10) F1 anti-DA/Sn (anti-H-2Kq)) were kindly provided by Dr. John G. Ray, Jr., Research Resources Branch, National Institutes of Health, Bethesda, Md.

Virus Inoculation. Under anesthesia, 0.05 ml of the virus preparation was inoculated into each lobe of the thymus of 3- to 6- wk-old mice.

Absorption Studies. The expression of H-2D and H-2K antigens in normal and RadLV-infected thymocytes was determined as previously described (2) by measuring the ability of these cells to absorb appropriate alloantisera.

35S-Methionine Labeling of Lymphocytes and One-Dimensional Sodium Dodecyl-Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analyses. Animals were killed by cervical dislocation and their spleens, or solid tumors growing intraperitoneally excised and placed in phosphate-buffered saline (PBS). Single cell suspensions were prepared by gentle teasing and pipetting. Labeling with 35S-methionine and extraction with 0.5% NP-40 was done as previously described (4). The cell lysate was then centrifuged at 100,000 g for 1 h. The supernate was aliquoted and stored at -70°C until further use. For immunoprecipitation portions from 5 × 10⁶ cells were cleared of labeled immunoglobulins with goat anti-mouse Ig (GAMIG) and carrier normal mouse serum at equivalence. The resulting precipitate was then centrifuged at 8,000 rpm for 30 min. The supernate was then incubated at 37°C for 30 min with specific or control antiserum. GAMIG was then added again and the samples incubated at 4°C overnight. The precipitate was then collected by centrifugation at 8,000 rpm for 20 min, washed at least four times in 1% Triton-X-100-1% deoxycholate in PBS, pH 7.4, dried, and then subjected to SDS-PAGE according to the method of Witte and Weissman (5).

Cultures. All RadLV-transformed tissue culture lines were adapted from RadLV infected, leukemic animals. Lymphoid tissues were removed and placed in culture at a concentration of 2-4 × 10⁶ cells/ml. Cultures were split and fed after 2 d. Afterwards, daily observations of the cultures were carried out to feed the cells as required while always keeping a portion of the conditioned growth media present. The first signs of successful adaptation of the cultures was the formation of an epithelial sheet with clusters of round cells throughout. After several weeks, the latter cells became autonomous and the feeder-layer unnecessary. Success rates in adapting lymphoid tumors and culture varied between 30 and 60%.

BW5147 cells were originally obtained from the Salk Institute, San Diego, Calif. and have been maintained independently since 1975. Their karyotype and growth conditions have been described previously (6).

Preparation of Effector Cells, 51Cr-Labeled Target Cells, and Assay for Cell-Mediated Cytotoxicity. Cells were prepared and cell-mediated lympholysis activity measured as previously described (6), except that cells used for this test were grown in RPMI-1640 with 25 mM Hepes (Grand Island Biological Co., Grand Island, N. Y.). The assay was also carried out in RPMI-1640. Special care was given to the use of fresh Na 51Cr (Amersham Corp., Arlington Heights, Ill.) for labeling target cells. Incubation times varied from 4 to 12 h.

Antiserum and Complement-Mediated Cytotoxicity. Spleen cells were prepared with gentle teasing in Dulbecco’s modified Eagle’s medium (DMEEM) and 5% FCS, centrifuged, counted, and aliquoted in tubes at a concentration of 20 × 10⁶/tube. They were then centrifuged and resuspended in 1 ml of 1/5 dilution of the appropriate antiserum or control serum (diluted in
DMEM + 5% fetal calf serum (FCS). After 30 min at room temperature, they were spun down, and resuspended in 3 ml of 1/9 dilution (diluted in DMEM) of agarose-adsorbed rabbit complement. After 45 min incubation at 37°C, cells were pooled (if more than one tube of 20 x 10^6 cells was treated with a given serum), counted to assess percent cytotoxicity, centrifuged, washed three times, and recounted.

Results

Resistance to Syngeneic RadLV Tumor Cells Mimics H-2D Control of RadLV-Induced Neoplasia. It has been previously shown (19) that if RadLV infected, transformed lymphocytes (B10.S) are injected into (B10.S × B10.G) F1 (susceptible to the virus) or (B10.S × B10.S(7R)) F1 (resistant) mice, the latter survive much longer than the former. Likewise (B10.G × B10.S) (susceptible) F1 mice survive a shorter time after inoculation of B10.G RadLV-transformed cells than do (B10.G × B10.T(6R)) F1 (resistant) animals. In this sense, the observed resistance to tumor cells is consistent with the known observations about H-2D control of RadLV-induced neoplasia.

Initial attempts to demonstrate cell-mediated immunity (CMI) against the injected B10.S and B10.G tumor cells failed, even in the hybrids surviving the tumor challenge.

H-2-Negative, RadLV Transformed Cells, Re-Express H-2 Antigens after In Vivo Growth. It has been shown by absorption analysis that RadLV-induced tumor cells adapted to culture, appear to show no detectable expression of H-2 antigens (2). Because H-2 antigens seem to be required for cell-mediated killing (7-9), difficulties with the in vitro assay for CMI may have resulted from the use of tissue culture adapted RadLV transformed cells as targets. Therefore, an attempt was made to determine whether H-2 modulation occurred if these cells were grown in vivo in normal, nonleukemic animals.

When syngeneic RadLV transformed cells are injected intraperitoneally into resistant and susceptible animals, they do not grow as an ascites tumor. In susceptible mice, tumor cells initially invade the spleen and then multiply in other lymphatic organs. However, in resistant mice tumor cells grow in the spleen and lymphatic organs to a lesser extent and predominantly as a solid peritoneal tumor. Tumor cells multiply slower in resistant than in susceptible mice, and are often completely eliminated after some initial growth in the former animals.

Fig. 1 shows the results obtained when 5 x 10^6 RadLV transformed, tissue culture grown B10.G cells were injected intraperitoneally into (B10.G × B10.S) F1 (susceptible) and (B10.G × B10.S(7R)) F1 (resistant) mice and spleens of three to four mice of each type removed at various intervals to measure H-2 antigen re-expression on the injected cells. (B10.G × B10.S) F1 spleen cells were incubated with anti-D^q serum and complement and (B10.G × B10.S(7R)) F1 spleen cells were incubated with anti-D^q serum. Because the tumor cells were D^q positive, neither serum should react with the growing cells, and only host cells should be lysed by the antiserum plus complement treatment. However, treatment of (B10.G × B10.S) F1 and (B10.G × B10.S(7R)) F1 spleen cells with anti-D^q serum plus complement should lyse 100% of the cells. Because the tumor cells were D^q positive, neither serum should react with the growing cells, and only host cells should be lysed by the antiserum plus complement treatment. However, treatment of (B10.G × B10.S) F1 and (B10.G × B10.S(7R)) F1 spleen cells with anti-D^q serum plus complement should lyse 100% of the cells, because all host cells and tumor cells should be killed (provided that the tumor cells reexpress H-2D^q). It can be seen that in the case of (B10.G × B10.S) F1 cells, anti-D^q serum kills 95% of the cells on day 0, but kills fewer and fewer cells on subsequent days, as the injected tumor cells multiply in the spleen. Killing by anti-D^q sera on the other hand, starts out at 96% on day 0 and declines initially at the same
rate as killing by anti-D^a, but begins to kill a larger percentage of the cells after day 9. The same pattern can be observed in (B10.G × B10.S(7R)) F1 mice. However, the percentage of spleen cells of these mice that can be killed with anti-D^a after day 9 is much higher than the percentage of spleen cells that can be killed with this serum for (B10.G × B10.S) F1 mice.

These findings strongly suggest that phenotypically H-2-negative tumor cells can proliferate in vivo to give rise to tumor cells which express the appropriate H-2 antigens. However, the above experimental procedures involve simultaneous comparison of killing by several antisera; and is less desirable than more direct measurements. Fortunately, RadLV transformed cells often grow as solid peritoneal tumors in resistant mice. These tumors are composed almost entirely of donor cells and few host cells. Because little contamination, if any, by host cells is encountered, direct assay of H-2 re-expression can be carried out on the growing tumor cells. By use of two additional techniques, it has been possible to demonstrate directly that H-2 antigen re-expression occurs for cells derived from the injected cells, as they multiply in the peritoneal cavity. For example, when a fixed number of tumor cells is recovered and tested at various intervals after inoculation, their anti-H-2 absorbing capacity gradually increases (data not shown). Likewise, the ability of these cells to bind H-2 antibodies, as detected by a two-step radioimmunoassay, markedly increases as their exposure to the in vivo environment lengthens (data not shown).

As a further check, the re-expressed antigens were compared by SDS-PAGE with normal spleen cellular H-2 antigens. Spleen cells, tissue culture adapted B10.T(6R), and in vivo grown B10.T(6R) cells from a solid peritoneal tumor were incubated with ^35S-methionine for 3 h. After this step, immunoprecipitation of NP-40 lysates was done with H-2 alloantisera and goat anti-mouse IgG, and the resulting precipitates were submitted to SDS-PAGE analysis and compared with one another (Fig. 2). Panel A shows an important fact. RadLV transformed tumor cells growing in vitro
not only fail to express H-2 antigens as detected by absorption analysis (2), but also cannot be shown to synthesize these antigens by the present scheme. It is possible that synthesis of these antigens does occur, yet degradation takes place immediately after the nascent polypeptide chains are formed, yielding no detectable product, or that a nonantigenic polypeptide precursor is formed and remains uncleaved. Panel C is a control spleen preparation. The usual 45,000 and 12,000 mol wt pattern, associated with H-2 antigens and the mouse analogue of β2 microglobulin, respectively, is obtained. Transformed B10.T(6R) tumor cells growing in vivo (panel B) show a similar pattern as the spleen cells although the amount of material precipitated from comparable cellular extracts appears to be smaller.
CMI Activity against RadLV Transformed Cells Can Be Detected Only by Using H-2-Positive Target Cells. That antigen re-expression is important in the effectiveness and demonstration of CMI immunity is apparent from Fig. 3. When $1.25 \times 10^6$ spleen cells from resistant animals, injected 12-18 d before, are incubated with the same tissue culture grown cells ($5 \times 10^6$) as they were inoculated with, no CMI activity is detectable. On the other hand, when these same spleen cells are incubated with $5 \times 10^4$ RadLV tumor cells grown in vivo for 10 d, CMI activity is clearly demonstrable. The CMI response appears to be specific because neither BW5147 cells (H-2k) nor normal spleen cells (H-2a) are lysed by the effector cells. They also fail to lyse EL-4 (H-2Db), which were derived from a C57BL/6 mouse (data not shown).

A technically more difficult experiment is shown in Fig. 4. Here tumor cells growing in the spleen of hybrid animals injected for 10 d are recovered after elimination of normal cells with antiserum to the parental H-2 haplotype opposite to that expressed by the tumor plus complement. The recovered cells are then labeled with $^{51}$Cr and used as targets for killer cells derived from hybrid mice, injected 12-18 d before with tumor cells. Panel A shows incubation of (B10.G × B10.S(7R)) F1 (resistant) killer cells with in vivo and in vitro grown B10.G cells and panel B incubation of these same targets with killer cells derived from (B10.G × B10.S) F1 (susceptible) mice injected 12-18 d previously with B10.G tumor cells. In addition to demonstrating a requirement for H-2 antigens to obtain killer cell activity, the results show that resistant mice develop effective killer cells to a tumor challenge to which susceptible mice are not capable of responding effectively.

Resistant Mice Develop Greater Numbers of Effector Cells When Inoculated with RadLV Than Do Susceptible Mice. At various intervals 3-6 wk of age B10.G (susceptible) and
RadLV infected thymocytes from resistant mice can elicit strong CML responsiveness when used to challenge normal F1 mice. The results so far suggest that H-2 antigens are required for successful killing of target cells, because in vitro grown H-2-negative cells are not lysed. In addition, viral antigens are required because normal, H-2-positive spleen cells do not serve as targets. But none of these studies demonstrates that virus infected thymocytes displaying high levels of H-2 are better immunogens than normal thymocytes.
Fro. 5. Resistant mice develop effective CMI when inoculated with RadLV although susceptible mice do not. Weanling B10.G (susceptible) and B10.T(6R) (resistant) mice were inoculated intrathymically with RadLV at various times. These mice were then assayed simultaneously for CMI as described in the text.

An experiment designed to test the latter concept is shown in Fig. 6. (B10.S X B10.S(7R)) F1 mice were inoculated with thymocytes from B10.S (bottom panel) or B10.S(7R) (top panel) mice untreated, or previously inoculated with RadLV at various intervals. The ensuing CML response against in vivo passaged B10.S or B10.S(7R) RadLV transformed cells was then measured 12 d later. The results clearly indicate that virus infected thymocytes displaying high levels of H-2 are better immunogens than normal thymus-derived lymphocytes.

Discussion

The findings presented in this report indicate that the genetics of resistance to syngeneic RadLV-induced tumor cells are identical to H-2D control of RadLV-induced leukemogenesis. These findings are suggestive of a role for H-2D gene(s) at some point after malignancy has ensued. This type of resistance would be expected to be immunological. Several observations are in accord with this expectation. First, CMI against RadLV transformed or infected cells can be detected with ease when H-2-positive target cells are used in the cell-mediated lympholysis (CML) assay. Second, resistant mice develop greater numbers of effector cells when infected with RadLV than do susceptible mice. Third, the previous observation that rapid increases in cellular synthesis and cell-surface expression of H-2 antigens are detectable immediately after virus inoculation has suggested that altered expression of H-2 antigens may play a significant role in the mechanism(s) of host defense to virus infection. This concept is strengthened by the findings documented in Fig. 6; namely injection of normal (uninfected) thymocytes into syngeneic recipients of resistant or susceptible
H-2 type does not stimulate a CML response. However, injection of RadLV infected thymocytes from resistant mice produces a vigorous CMI response, and such thymocytes elicit the strongest response at a time when both H-2 and viral antigen expression is elevated. By contrast, injection of infected thymocytes from susceptible mice, which express viral antigens, but low levels of H-2 antigens, does not stimulate a CML reaction. In addition, target recognition appears to be specific for H-2D\(^d\) antigens because F1 mice challenged with infected B10.S(7R) thymocytes can lyse B10.S(7R) targets (D\(^d\)), but not B10.S target (D\(^d\)), even after both targets have been passaged in vivo and express H-2 antigens.

Preliminary experiments indicate that transfer of thymocytes infected with RadLV for a short time (e.g. 3 wk), from resistant or susceptible mice, into irradiated adult, syngeneic F1 mice leads to death from tumorigenesis in a relatively short time (D. Meruelo, unpublished observations). It is unlikely that such death results from the induction of leukemia by virus present in transformed cells, because the progress of disease is too rapid, and RadLV does not lead to leukemia when injected into adult
mice. The likely conclusion is that preleukemic cells exist in the thymus of both resistant and susceptible mice and that transfer to the irradiated host environment permits such cells to escape the immunological control that prevents their growth in unirradiated, resistant mice.

It has been shown here that the failure to detect differences in anti-RadLV CMI between resistant and susceptible animals was due to the absence of H-2 determinants on the target cells used. A change in phenotypic expression from H-2+ to H-2− accompanies overt leukemia and can be reversed by in vivo passage by RadLV transformed cells through healthy, syngeneic recipients. H-2 antigen modulation has been previously observed by Boyse et al. (10, 11). In these studies, an inverse relationship was observed in cells undergoing antigenic modulation between the expression of TL and H-2D antigens. It has been suggested that TL antigens derived from a viral genome integrated in chromosome 17 (12). In this regard, two observations are relevant. First, tissue culture adapted, RadLV-transformed cell lines produce substantial levels of thymotropic virus, and are H-2 negative, but after in vivo passage these cells show markedly reduced virus production and full expression of H-2 antigens. In addition, it has been shown (2) that from the early stages of the disease, there is an inverse relationship between viral and H-2 antigen expression. A reduction in virus production could be explained by antibody or cellular elimination or reduction of RadLV. However, there is some evidence that H-2D gene(s) regulate virus production (13). In view of these findings, the notion that a viral genome is integrated in chromosome 17 is provocative and should be pursued further.

The data presented here are of further relevance in view of two established observations regarding type-C RNA virus-induced leukemia. First, it is well known that murine type-C RNA virus must be inoculated in neonatal or very young mice for successful leukemogenesis. Second, a great many of these viruses exhibit preferential tropism for the thymus. This is best exemplified by RadLV, which is usually injected intrathymically for optimal leukemia incidence, and causes a disease which has as principal characteristic the development of thymomas (14). In view of the importance of H-2 antigens for T-cell killing (7-9), these two facts could be explained if virus infection of neonatal or thymocytes (low H-2 bearing cells) is more successful than infection of adult or mature cells because cells having high levels of H-2 and virus antigen trigger strong CMI and become rapidly eliminated.

The very rapid and dramatic increases in expression of H-2 antigens on thymocytes of resistant mice immediately after virus injection may represent an evolutionarily developed defense mechanism. This mechanism of defense would be expected to be of particular importance because it occurs in the thymus. Zinkernagel et al. (15) have recently provided evidence that the thymus is the site where cell-cell recognition is learned.

There is one observation that cannot be completely explained by these results. This is the finding that F1 mice of resistant H-2 type survive a tumor inoculation longer than do F1 mice of susceptible H-2 genotype. Because tumor cells growing in both F1s appear to re-express H-2 antigens after a few days of in vivo growth, the inability of susceptible mice to reject the tumor cells and the ability of resistant mice to, at least, survive longer, raise the possibility that an Ir gene important in resistance to RadLV-induced neoplasia may be operative in the H-2D region. If so, H-2q/d of H-2q/d F1 mice would be high responders, whereas H-2q/k F1 would be low
H-2 ANTIGEN EXPRESSION AFFECTS RESISTANCE TO LEUKEMA

responders. This observation requires additional study.

The present studies need to be expanded to encompass other type-C RNA viruses. Some investigators have reported similar effects on H-2 antigen expression by Friend (16) and AKR viruses (17), but a more detailed study is required. If similar changes in HLA expression at various stages of leukemogenesis could be demonstrated (suggested by reported instances in which investigators were unable to type leukemic cells [18]), these findings would have very significant clinical implications.

Summary

Resistance to neoplasia caused by radiation-induced leukemia virus (RadLV) is mediated by gene(s) in the H-2D region of the major histocompatibility complex. The previous observation that rapid increases in cellular synthesis and cell-surface expression of H-2 antigens are detectable immediately after virus inoculation has suggested that altered expression of H-2 antigens may play a significant role in the mechanism(s) of host defense to virus infection. This concept is supported by the following observations. First, cell-mediated immunity against RadLV transformed or infected cells can be detected with ease when H-2-positive target cells are used in the cell-mediated lympholysis (CML) assay. (Although RadLV transformed cells obtained from overtly leukemic animals and maintained in tissue culture are H-2 negative, these cells can regain their H-2 phenotype by in vivo passage in normal animals. The H-2-negative cells are poor targets in a CML assay.) Second, resistant mice develop greater numbers of effectors when infected with RadLV than do susceptible mice. Third, injection of normal (uninfected) thymocytes into syngeneic recipients of resistant or susceptible H-2 type does not stimulate a CML response. However, injection of RadLV infected thymocytes from resistant mice produces a vigorous CMI response, and such thymocytes elicit the strongest response at a time when both H-2 and viral antigen expression is elevated. By contrast, injection of infected thymocytes from susceptible mice, which express viral antigens, but low levels of H-2 antigens, does not stimulate a CML reaction.

These findings may explain the easier induction of leukemia found by many investigators when virus is inoculated into neonatal mice and the preferential thymus tropism of some oncogenic type-C RNA virus. Cells expressing very low levels of H-2, such as thymocytes, may serve as permissive targets for virus infection because they lack an important component (H-2 antigens) of the dual or altered recognition signal required to trigger a defensive host immune response.

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