DIFFERENTIAL INDUCTION OF H-2K VERSUS H-2D CLASS I MAJOR HISTOCOMPATIBILITY ANTIGENS BY RECOMBINANT γ INTERFERON

Lack of K^k Augmentation in a Leukemia Virus–Induced Tumor Is Due to a cis-dominant Effect

By WILLIAM R. GREEN, ROBERT F. RICH, AND CAROL BEADLING

From the Department of Microbiology and the Norris Cotton Cancer Center, Dartmouth Medical School, Hanover, New Hampshire 03756

It is widely accepted that the presence and the levels of expression of class I MHC antigens on virus-infected and tumor cells are of crucial importance for recognition by T lymphocytes, particularly cytolytic T lymphocytes (CTL). In that tumors frequently exhibit low constitutive levels of MHC expression, the ability of the interferons, especially IFN-γ, to upregulate MHC expression may thus become critical to successful immune recognition and elimination of such unwanted cells. On the other hand, the ability of a neoplastic cell to eliminate class I expression or to lower constitutive levels of class I–restricting elements for CTL, coupled with a resistance to the enhancing effects of IFN, might provide an escape mechanism from immune recognition that would confer a selective advantage for tumor establishment.

We have been interested in the interaction of specific antiviral CTL with tumors induced by AKR/Gross murine leukemia viruses (MuLV)[1] with respect to the recognition of both viral antigens and the H-2K class I–restricting element used in this system (1–4). Recently, we identified two MuLV⁺ tumors of the high leukemia incidence H-2ᵏ haplotype that showed an unusual phenotype with regard to class I expression after IFN-γ treatment. In contrast to normal cells and most tumor lines, where there is a coordinate induction or augmentation of the expression of class I antigens, in these two tumors only H-2Dᵏ, not H-2Kᵏ expression was augmented by IFN-γ (5) or IFN-α (Green, W. R., et al., unpublished observations). The lack of induction of K^k was found not to be dependent on a particular dose of IFN-γ or time of treatment, and the low but significant constitutive levels of K^k expression argued against a deletion or general inactivation of the K^k gene. In addition, the augmentation of D^k expression indicated that IFN-γ was binding to the cells, resulting in signal transmission.

The present study addresses the mechanism behind this differential induction of class I genes in one of these two tumors, the AKR SL3 line. Although IFN signaling of class I genes is generally believed to occur primarily at the transcriptional level (6, 7), IFN regulation appears to be complex. A number of cis-acting regulatory sequences have recently been described for IFN-γ and/or IFN-α/β (8–10). In many cases, however, the magnitude of the effects dictated by these sequences only partially accounted for the increases in cell surface expression of class I protein. In addition, there are several reports of trans-acting factors that are involved in the control of class I expression (11, 12). In this communication we report our initial attempts to define the mechanism by which the K^k gene of the AKR SL3 line is resistant to IFN-γ augmentation by considering cis-dominant vs. trans-acting alterations. To differentiate between these two possibilities, T-T tumor hybrid cell lines were constructed between the AKR SL3 line and a T tumor line of a distinguishable H-2 haplotype that shows normal augmentation of both K and D class I antigens in response to IFN-γ treatment.

Materials and Methods

IFN-γ. Murine rIFN-γ produced by *Escherichia coli* that had been transfected with the DNA coding sequence for IFN-γ was kindly supplied courtesy of Dr. H. Michael Shepard, by Genetech, Inc., South San Francisco, CA, and was used at a concentration of 100 U/ml in tissue culture medium (TC).

Cell Culture Media. All parental tumors and hybrids were passaged in TC consisting of RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Hyclone Laboratories, Logan, UT), antibiotics, 2 mM glutamine, and 50 μM 2-ME (Sigma Chemical Co., St. Louis, MO).

H-2–specific Antibodies. HB19, specific for H-2D^b; and HB41, specific for H-2K^b; are IgM mAbs used as supernatant and concentrated supernatant, respectively, whose corresponding hybridomas were obtained from the American Type Culture Collection, Rockville, MD. Hybridoma cells for mAb 15-5-5S, IgG2a anti-H-2D^k, were kindly provided by Dr. K. Ozato (National Institutes of Health, Bethesda, MD). mAb 11-4.1, IgG2a anti-H-2K^k, was used as an ascites preparation and was kindly provided by Dr. R. C. Nowinski (Genetic Systems, Seattle, WA). IgM SCC (anti–human small cell carcinoma of the lung), IgM 35/12 (anti-DNP, gift of Dr. R. Noelle, Dartmouth), and IgG2a OKT8 were used as negative control mAbs.

Drug Marking of Parental Tumor Cells and T Tumor Fusions. E ♀ K1, a tumor of C57BL/6 (H-2^b) origin (13), was drug marked by growing the cells in TC medium containing dialyzed FCS and 67 μg/ml of 5-bromo-deoxyuridine (BUdR, Sigma Chemical Co.). A drug-resistant clone obtained by limiting dilution, E ♀ K1-cl.B2-BUdR^R, was used as the H-2^b parental partner in all fusions. SL3, a tumor line of AKR (H-2^k) origin (13), was drug marked with 1.5 μg/ml of 8-azaguanine (AZ, Sigma Chemical Co.), yielding the AKR SL3-AZ^R line used in fusions 1 and 2. This line was subsequently exposed to 100 U/ml of IFN-γ for 6 d followed by a one cell per well sterile sort for optimal D^k antigen expression (for details on sorting see analogous hybrid K^b sorting discussed below), yielding clones including AKR SL3-cl.F-AZ^R, which was used as the H-2^k parental tumor in fusion 3. For hybrid formation, between 4×10^6 and 1×10^7 T tumor fusion partner cells were used at 1:1 ratio using PEG 1500 (Boehringer Mannheim Biochemicals, Indianapolis, IN). Selection for hybrids was carried out in TC supplemented with hypoxanthine, aminopterin, and thymidine (HAT, Sigma Chemical Co.).

Indirect Immunofluorescence, Sorting, Two-Color Analysis. To ensure their general receptiveness to IFN-γ signaling of MHC expression, T tumor hybrids from fusions 1 and 2 were exposed to 100 U/ml of IFN-γ for 6–7 d with daily medium change. Whereas all hybrids tested showed significant augmentation of D^k and K^b expression, confirming that chromosome(s) 17 of both parental types were present, the brightest 0.1–0.5% of hybrids

expressing K^b were sterilely sorted to one cell per well. After 10–14 d clones were sequentially weaned from HAT to TC medium, and in some cases underwent an extensive two color-analysis using a cytofluorograph (Ortho Diagnostic Systems Inc., Westwood, MA) (Table I). K^b expression was detected using mAb HB41 followed by FITC-conjugated goat anti–mouse μ chain–specific serum (Boehringer Mannheim Biochemicals). D^k expression was detected using mAb 15-5-5S followed by R-Phycoerythrin (PE)-conjugated goat anti-mouse γ chain–specific serum (Southern Biotechnology Associates, Inc., Birmingham, AL). Correction factors were used that effectively eliminated green color bleeding into red or vice versa. For analysis of H-2K^b, D^b, K^k, and D^k antigen expression without and after IFN-γ exposure for 6–7 d, hybrid and parental tumor lines were incubated sequentially with various dilutions of control or anti-H-2 mAb followed by FITC-labeled goat F(ab')$_z$ anti–mouse IgG, IgA, IgM, H chain– and L chain–specific antiserum (Cooper Biomedical, Inc., Malvern, PA) before analysis on the cytofluorograph.

Results and Discussion

To construct hybrids, E ♀ K1-cl.B2-BUdR^R, a BUdR-resistant subline of the C57BL/6 (H-2^b) thymoma, was fused with the AZ-resistant AKR SL3-AZ^R(H-2^k) variant with PEG followed by HAT selection. These parental lines were unable to grow in the opposite drug or in HAT-containing medium. Two-color immunofluorescence and flow cytometry were used to confirm the hybrid nature of the fused cells and their retention of chromosomes encoding both parental MHC haplotypes. In contrast to the parental tumor lines, a substantial percentage (up to 99% of cells for the five hybrids tested in Table I, and all additional hybrids tested, including fusion 3 below), expressed both K^b and D^k antigens. This was particularly apparent for some hybrids after their low-to-moderate parental levels of constitutive expression were boosted by IFN-γ treatment. Indeed, 97–99% of the cells of these hybrids then simultaneously expressed both easily detectable K^b and D^k. The enchancement of MHC expression by all hybrids after IFN-γ treatment also confirmed their receptiveness to IFN activation. Mixtures of hybrid cells stained separately for their K^b or D^k antigens or mixtures of parental tumor cells stained for their respective class I antigens resulted in minimal percentages of cells scored as "double positives." In addition, the failure to detect both K^b and D^k on the parental tumors confirmed the lack of crossreactivity of the monoclonals used.

Hybrid clones from two independent fusions were next treated with IFN-γ, and the expression of all four class I MHC antigens was assessed separately by one-color flow cytometric analysis. IFN-γ treatment resulted in substantial increases in the percentages of positive cells and/or in the total mean fluorescence for D^b, K^b, and D^k antigens for all hybrids tested (Table II). In sharp contrast, the expression of K^k by the hybrids, like that by the parental AKR SL3 line, selectively showed little or no change after IFN-γ treatment. In particular, the stimulation of K^b expression was often dramatic, suggesting that if an IFN-γ–triggered, positive-acting, K locus–specific factor existed, it should have been readily available to augment K^k expression. In addition, it should be noted that, although the constitutive levels of the different class I antigens varied, frequently the constitutive level of K^k expression was similar to that of the augmentable K^b and/or D^k genes. This pattern of results was remarkably reproducible, as shown by the summarized fold increase data for Exp. 2 (Table II). In

TABLE I

Flow Cytometric Evidence That HAT-selected Tumor Cells Are Hybrids and Express Both Inducible H-2^b and H-2^k Class I MHC Antigens

Hybrid tumors	IFN-γ	K^b		D^k		K^b, D^k
		Positive	TMFI	Positive	TMFI	Positive*
		%		%		%
F1-1-E	−	61	17	38	18	74
	+	99	142	97	83	99
F2-1-A	−	32	9	91	49	49
	+	99	85	99	150	98
F2-1-D	−	99	60	100	130	99
	+	ND	ND	ND	ND	ND
F2-1-F	−	46	11	86	81	35
	+	98	64	100	152	97
Parental tumors						
E ♀ K1-cl.B2-BUdR^R	−	36	12	2	3	0
	+	99	119	0	1	0
AKR SL3 AZ^R	−	0	1	60	35	1
	+	0	1	92	98	6

Hybrid (fusion 1) and hybrid and parental (fusion 2) tumor cells were stained for K^b and D^k antigen expression simultaneously, both without and after exposure to 100 U/ml IFN for 6–7 d. The percent positive cells and their total mean fluorescence intensity (TMFI, linear scale 0–200) are listed. Negative control staining with isotype-matched mAbs averaged 4.2%.

* The percent of cells, which were plotted to points within a region of histogram P4 (HP4), where both K^b and D^k antigens were detected. For fusion 1 this region was defined by mixing hybrids stained with either anti-H-2K^b or H-2D^k antibodies at a 1:1 ratio whereby ≤7% of cells were plotted to region HP4. For fusion 2, a mixture of the two parental lines, stained with anti-H-2K^b and H-2D^k simultaneously followed by corresponding second antibodies, were plotted such that <6% of the cells fell within region HP4. For more details see Materials and Methods.

addition, the three other hybrids from fusions 1 and 2 that were studied also showed the same differential lack of augmentation of K^k expression, consistent with a *cis*-acting alteration.

For fusions 1 and 2 we used a drug-marked population of AKR SL3 tumor cells in an attempt to utilize a source of cells as related as possible to the original AKR SL3 line, with which we demonstrated differential class I augmentation by IFN-γ (5). Because of possible heterogeneity in the AKR SL3 line, which had not been recently cloned, it was difficult to quantitatively compare the hybrid clones of fusions 1 and 2 with this H-2^k parent. That is, each hybrid resulted from a fusion of the E ♀ K1 partner with a single AKR SL3 cell, but there was no way to identify that particular AKR SL3 parental cell per se for comparison purposes. Even though the number of hybrids analyzed, all with similar IFN-γ responsiveness, argued against a skewing of results due to a nonrandom selection of H-2^k fusion partners, a third fusion was performed with a clonal source of AKR SL3 AZ-resistant cells, AKR SL3-cl.F-AZ^R. Several resulting hybrids were tested after IFN-γ treatment (Table III). Again, all hybrids showed substantial and repeatable increases in all class I MHC antigens except K^k, confirming our observations above. Five additional hybrids derived from fusion 3 were also tested with a very similar pattern of reproducible results (data not shown).

These data, obtained through the detailed analysis of 16 hybrid cell lines

TABLE II

IFN-γ Induced Augmented Expression of D^b, K^b, and D^k, But Not K^k, by Hybrids of the AKR SL3-AZ^R and E♀K1-cl.B2-BUdR^R Tumors

Hybrid tumor clones	Antigen	IFN-γ	Positive*	Total mean fluorescence		Exp. 2
				Change in TMFI‡	Fold increase§	
			%			
F1-1-A	D^b	−	60			
		+	86	118	2.5	3.5
	K^b	−	8			
		+	37	106	20.3‖	5.2
	D^k	−	25			
		+	66	74	3.4	6.4
	K^k	−	15			
		+	27	6	1.3	≤1
F1-1-E	D^b	−	87			
		+	97	112	1.6¶	2.4
	K^b	−	50			
		+	96	319	5.4	12.8
	D^k	−	71			
		+	96	158	2.5	8.3
	K^k	−	24			
		+	29	0	≤1	≤1
F2-1-A	D^b	−	84			
		+	99	374	3.4	3.0
	K^b	−	26			
		+	99	460	15.0	15.8
	D^k	−	5			
		+	99	220	5.2	5.5
	K^k	−	39			
		+	36	−9	≤1	≤1
F2-1-D	D^b	−	99			
		+	100	190	1.6¶	1.2¶
	K^b	−	94			
		+	99	318	2.3	2.2
	D^k	−	96			
		+	99	265	2.5	1.2¶
	K^k	−	35			
		+	24	−11	≤1	≤1
F2-1-F	D^b	−	90			
		+	98	201	2.1	1.7¶
	K^b	−	38			
		+	97	299	7.0	6.8
	D^k	−	58			
		+	97	190	3.8	3.1
	K^k	−	43			
		+	40	−3	≤1	≤1

TABLE II (Con'd)

| Hybrid tumor clones | Antigen | IFN-γ | Exp. 1 | | | Exp. 2 |
| | | | Positive* | Total mean fluorescence | | |
				Change in TMFI[‡]	Fold increase[§]	
Parental Tumors						
E9K1-cl.B2-BUdR[R]	D[b]	—	97			
		+	100	170	1.7[¶]	ND
	K[b]	—	84			
		+	100	394	4.1	
AKR SL3-AZ[R]	D[k]	—	71			
		+	92	228	3.4	ND
	K[k]	—	45			
		+	45	0	≤1	

For information on the parental hybrids see Materials and Methods.

* The percent of cells staining positive relative to isotype matched negative control mAb staining, which averaged 4.3% of total cells.

[‡] The change in total mean fluorescence was deduced using the formula $(E_i - C_i) - (E_c - C_c)$ where E_i is TMFI of induced experimental cells (i.e., anti-H-2 mAb), C_i is TMFI of induced control cells (i.e., isotype-matched control mAb), E_c is TMFI of constitutive experimental cells (i.e., anti-H-2 mAb), C_c is TMFI of constitutive control cells (i.e., isotype-matched control mAb).

[§] The fold increase in TMFI was deduced using the formula $(E_i - C_i)/(E_c - C_c)$. A value of ≤1 represents a decrease or no change in TMFI with exposure to IFN.

[‖] A large IFN-induced change in TMFI sometimes resulted in a very large fold increase when the constitutive level of expression was low.

[¶] Conversely, if constitutive expression was high, this might yield a lower fold increase although the change in TMFI might still be very substantial.

derived equally from fusions using the original AKR SL3 line or a clone of the latter, were consistent with the existence of an alteration cis to the noninducible K^k gene. Thus, there was no support for the absence of a K locus–specific, trans-acting, positive factor as the reason for the unusual differential class I induction phenotype of AKR SL3. The results are also inconsistent with the presence of a negative trans-acting regulatory factor. Indeed, the presence of such a K locus–specific factor in the AKR SL3 line and thus in the hybrids would have inhibited or tempered the IFN-γ–dependent augmentation of K^b gene expression in the hybrids. Clearly, K^b expression was substantially augmented in all hybrids tested. In addition, although such a negative trans-acting factor need not be linked to H-2 but could have been encoded on another chromosome that was selectively not maintained in all the hybrids, the loss of this putative factor should have resulted in augmentable K^k expression. The data clearly show that this was not the case. Thus, although more complicated mechanisms involving multiple alterations may be possible, the most straightforward explanation for the lack of augmentation of K^k expression in the AKR SL3 line is based on a cis-acting regulatory defect.

These results, together with our previous identification of a second tumor cell line that is also MuLV[+] and congenic for H-2[k] (5), are interesting in light of

TABLE III

Lack of Induction of Augmented K^k Expression by IFN-γ in Hybrids of the AKR SL3-cl.F-AZ^R and E ♀ KI-cl.B2-BUdR^R Tumors

Hybrid tumor	Antigen	IFN-γ	Positive	Exp. 1 Total mean fluorescence Change in TMFI	Exp. 1 Total mean fluorescence Fold increase	Exp. 2
			%			
F3-1-A	D^b	−	53			
		+	86	170	2.3	
	K^b	−	8			
		+	87	356	12.1	ND
	D^k	−	21			
		+	75	152	3.5	
	K^k	−	13			
		+	13	−1	≤1	
F3-1-E	D^b	−	44			
		+	98	360	4.3	2.5
	K^b	−	5			
		+	95	493	150.5*	12.8
	D^k	−	11			
		+	92	241	6.9	8.3
	K^k	−	20			
		+	17	−11	≤1	≤1
F3-1-I	D^b	−	41			
		+	97	373	5.3	2.5
	K^b	−	19			
		+	97	515	27.8*	17.1
	D^k	−	21			
		+	87	173	4.9	4.8
	K^k	−	21			
		+	34	−8	≤1	≤1
Parental tumors AKR SL3-cl.F-AZ^R	D^k	−	65			
		+	96	328	5.1	
	K^k	−	79			ND
		+	89	25	1.3	

For information on the AKR SL3-cl.F-AZ^R and E ♀ Kl-cl.B2-BUdR^R parental clonal tumor lines please see Materials and Methods. See Table II for explanation of headings. Isotype-matched negative control mAb staining averaged 6.5%.
* See footnote ‖ of Table II legend.

other studies on MHC expression by such tumors. Several studies have suggested aberrant expression of class I antigens by AKR tumors, particularly with respect to K^k (14–15). A tumor line described recently by Klyczek et al. (16) that does not express class I constitutively but also shows a differential induction of H-2D, not H-2K, antigens by IFN-γ is also Gross virus induced and of congenic

H-2^k origin. Whether the H-2^k haplotype and/or the presence of MuLV expression has any direct bearing on the differential induction phenotype is unclear, but it should be pointed out that Meruelo and colleagues have noticed a preferential integration of MuLV in the areas of histocompatibility loci, including the MHC (17, 18). Thus, it is possible that the integration of an MuLV in the regulatory region of the K^k gene has resulted in its failure to be stimulated by IFN-γ signals in the AKR SL3 line. The study of this tumor line may thus provide insights into tumor escape mechanisms and the mechanism of enhancement of class I MHC expression by IFN-γ.

Summary

T-T tumor hybrids were constructed between the AKR SL3 thymoma and an H-2-distinguishable thymoma cell line. Hybrids were stimulated with IFN-γ to determine whether the differential augmentation of H-2D vs. H-2K class I antigen expression by AKR SL3 in response to IFN-γ was due to effects cis or $trans$ to the noninducible K^k gene. For each of a large number of hybrids tested, the expression of H-2D^b, K^b, and D^k, but not K^k, was substantially enhanced by murine rIFN-γ. These results suggested that the lack of induction of the K^k gene was due to an alteration cis to K^k rather than to the presence or absence of K region–specific, $trans$-acting negative or positive factors, respectively.

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