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Immunology

Allogeneic H-2 antigen expression is insufficient for tumor rejection

(major histocompatibility complex antigen/class I molecule/tumorigenicity)

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ABSTRACT Murine A strain ($K^kD^dL^d$) sarcoma I (SaI) tumor cells have been transfected with a cloned H-$2K^b$ gene. The resulting clones (SKB clones) stably express high levels of a molecule that is serologically and biochemically indistinguishable from the H-2K^b antigen. SKB clones are not susceptible to cytotoxic T lymphocyte-mediated lysis by H-2K^b-specific bulk, cloned, or H-2K^b-restricted lymphocytic choriomeningitis virus-specific effectors. Survival times of A/J and B10.A mice challenged i.p. with the H-2K^b-expressing transfectants and the parental SaI cells are similar, suggesting that the presence of an allogeneic major histocompatibility complex class I antigen on the surface of this tumor line is insufficient for tumor rejection.

The mouse H-2 or major histocompatibility complex (MHC) encodes a collection of antigens that are of fundamental importance in an immune response. The classical H-2 class I or transplantation antigens are polymorphic cell-surface glycoproteins that consist of a 45-kDa heavy chain noncovalently associated with β_2-microglobulin (β_2m), a 12-kDa protein encoded outside the MHC (1, 2). The discriminatory capacity of cytotoxic T lymphocytes (CTL) for recognizing a particular viral or tumor-associated cell-surface antigen is restricted to cells that express self class I molecules (3). CTL can also exhibit a specificity for recognizing non-self class I molecules alone as target antigens. This phenomenon, termed allorecognition, is the basis for the immunological destruction of tissue allografts.

Several lines of evidence suggest that qualitative and quantitative changes in the expression of MHC class I products can influence *in vivo* tumor progression. The 1591 fibrosarcoma expresses MHC class I antigens that serve as CTL target antigens (4). MHC class I antigen-negative tumors (e.g., teratocarcinomas) proliferate in many allogeneic hosts (5–7). Increased tumor cell MHC class I antigen expression accompanies host resistance (8–10). Decreased expression or loss of class I antigen expression on a number of immunogenic murine tumors is correlated with increased tumorigenicity in syngeneic mice, presumably due to increased susceptibility to CTL (11–18).

The expression of transfected class I gene products in murine tumors allows for the direct assessment of the role of class I antigen expression in immunologically mediated tumor rejection. In the tumors studied to date, reexpression of the absent class I antigen results in reduction or abrogation of *in vivo* tumorigenicity (19–22). The present study was undertaken to more fully evaluate the role of MHC class I antigens in *in vivo* tumor growth. Murine A strain ($K^kD^dL^d$) sarcoma I (SaI) tumor cells were transfected with an H-$2K^b$ gene. Resulting H-2K^b antigen-positive transfectants were tested for tumorigenicity in syngeneic mice and for susceptibility *in vitro* to alloreactive H-2K^b-specific bulk and cloned CTL and H-2K^b-restricted virus-specific CTL. The expression of the H-2K^b alloantigen on SaI cells does not reduce the tumorigenicity of this tumor in syngeneic mice, suggesting that the presence of a MHC class I alloantigen on the surface of these cells is insufficient for tumor rejection.

MATERIALS AND METHODS

Mice. Mice were either purchased or bred and were maintained as described (7).

Cells. SaI is a chemically induced sarcoma of A strain mice originally obtained from The Jackson Laboratory. The EL4 (H-2^b) thymoma, the L929 (H-2^k) fibroblast cell line, and the P815 (H-2^d) mastocytoma were maintained as described (23). Mice were challenged i.p. with varying numbers of tumor cells in 0.5 ml of serum-free medium. The BM11-41 and BM10-38 H-2K^b-specific alloreactive CTL clones (24) were maintained in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, penicillin, streptomycin, fungizone (GIBCO), and 2% purified human interleukin 2 (Electro-Nucleonics, Silver Spring, MD) and passaged every 4 days with 5000-rad (1 rad = 0.01 gray) γ-irradiated (Gammator B, Kewaunee Scientific, Statesville, NC) C57BL/6J spleen cells at a 1:1 responder:stimulator ratio. Every 8 days and prior to use, dead cells were depleted from the cultures by centrifugation through Ficoll/Hypaque (Pharmacia).

Monoclonal Antibodies (mAb). The isotypes and specificities of the mAb used are given in Table 1. Antibodies were titrated and used as described (25).

Recombinant DNA Vector and Transfection Procedures. pGC101 was constructed by inserting a 10.5-kilobase (kb) *Eco*RI fragment containing the entire H-$2K^b$ gene from the C57BL/6Kh mouse (32) into the unique *Eco*RI site of the plasmid vector pSV2gpt (33). SaI cells were transfected by protoplast fusion (34). L929 cells were transfected by the calcium phosphate method (35). Cells were maintained in normal growth medium for 3 days following transfection and then transferred to selective medium. Selective medium contained xanthine (250 μg/ml), adenine (25 μg/ml), thymidine (10 μg/ml), amethopterin (2 μg/ml), and mycophenolic acid (25 μg/ml). SaI transfectants were expanded in selective medium and cloned by limiting dilution. A single serologically H-2K^b antigen-positive clone was selected and recloned by limiting dilution to yield the resulting SKB clones. L929 cell transfectants were cloned once by limiting dilution. The SKB and L929-K^b transfectants have been monitored intermit-

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Abbreviations: CTL, cytotoxic T lymphocyte(s); MHC, major histocompatibility complex; LCMV, lymphocytic choriomeningitis virus; β_2m, β_2-microglobulin; LDCC, lectin-dependent cellular cytotoxicity; mAb, monoclonal antibody(ies).

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Table 1. mAb used in this study

mAb	Isotype	Specificity	Reactive domain	Ref.
11-4-1	IgG2a	K^k		26
20-8-4	IgG2a	K^b, K^d, Qa2*	α1	27, 28, 29
28-13-3	IgM	K^b	α2	27, 28
28-14-8	IgG2a	D^b, L^d		27
34-5-8	IgG2a	D^d		30
19/178	IgG2a	Lyt 2.2		31
PG14.1	IgG2a	VSV G protein		

VSV, vesicular stomatitis virus.
*mAb 20-8-4 reacts with 46- to 50-kDa H-$2K^b$ and H-$2K^d$ class I molecules and the 41-kDa Qa-2 molecule (30).

tantly by fluorescence flow cytometry following passage in nonselective medium for 30 and 20 months, respectively. In all cases, H-$2K^b$ antigen expression has remained stable.

Immunofluorescence. Indirect immunofluorescence was performed as described (25). Stained cells were resuspended in Hepes/Hanks' buffer and analyzed on a Coulter Epics C flow cytometer (Coulter).

Immunoprecipitations. Cells were metabolically labeled for 60 min with 200 μCi (1 Ci = 37 GBq) of [^{35}S]methionine (New England Nuclear) at a concentration of 1×10^7 cells per ml in methionine-free medium (GIBCO). Cell lysates containing 5×10^6–1×10^7 cpm were immunoprecipitated as described (36) by using undiluted mAb ascitic fluid and protein A-Sepharose (Pharmacia). Immunoprecipitated material was analyzed by $NaDodSO_4$/polyacrylamide slab gel electrophoresis ($NaDodSO_4$/PAGE) on 12.5% acrylamide gels (37). Gels were treated with EN^{3}HANCE (New England Nuclear) for fluorography, dried, and exposed to XAR-5 film (Kodak) at −80°C.

***In Vitro* CTL Assays.** Anti-H-2 effector cells were generated, and ^{51}Cr release assays were performed as described (23). Effectors and targets for lectin-dependent cellular cytotoxicity (LDCC) were generated as described (38). Lymphocytic choriomeningitis virus (LCMV), UBC strain, was used for infection of target cells, and the generation of LCMV-specific primary CTL was performed as described (39). The % of specific ^{51}Cr release = 100× [(experimental release − spontaneous release)/(total release − spontaneous release)]. For LDCC assays, % of specific lysis = release in presence of Con A − release without Con A.

RESULTS

Transfected SaI Cells Stably Express H-$2K^b$ Antigen. SaI cells were transfected with the pGC101 construct containing the *H-$2K^b$* gene and selected for growth in hypoxanthine/aminopterin/thymidine (HAT) medium supplemented with xanthine and mycophenolic acid. Transfectants were tested for specific class I antigen expression by indirect immunofluorescence. As shown in Fig. 1 *f* and *i*, two transfectant clones, SKB3.1L and SKB3.1M, specifically express the H-$2K^b$ antigen (mAb 20-8-4). Equivalent staining of the SKB3.1L and SKB3.1M clones is seen with mAb 28-13-3 (data not shown). Neither untransfected SaI cells (Fig. 1*c*) nor SaI cells transfected with the pSV2gpt plasmid containing the *$B2m^b$* gene stain for H-$2K^b$ antigen (data not shown). H-$2K^b$ antigen reactivity is specific as seen by the absence of reactivity of the cells with the irrelevant mAb (19/178; Fig. 1 *a*, *d*, and *g*). Other SKB clones (SKB3.1O, SKB3.1E, SKB3.1D) show similar levels of staining with the 20-8-4, 11-4-1, and 19/178 antibodies (data not shown). pGC101-transfected L929 cells (L929-K^b) strongly express H-$2K^b$ antigen (Fig. 1*o*).

The H-$2K^b$ antigen expressed on SKB and L929-K^b cells has been analyzed and compared to H-$2K^b$ antigen of EL-4 cells. Cells were [^{35}S]methionine labeled and detergent solubilized, the MHC class I molecules were immunoprecipitated, and the resulting immunoprecipitates were analyzed by $NaDodSO_4$/PAGE. As shown in Fig. 2, mAb 20-8-4 precipitates 45-kDa and 12-kDa polypeptides from both the

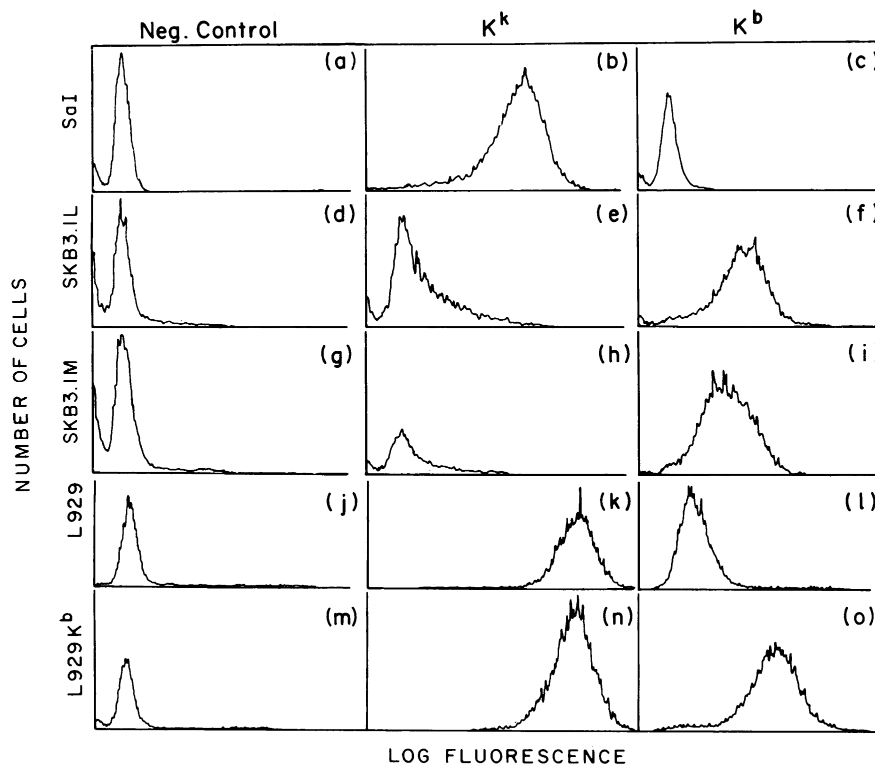

FIG. 1. Flow cytometry profiles of transfected SaI and L929 cells labeled by indirect immunofluorescence for H-$2K^b$ antigen. Each histogram represents 5×10^3 cells. K^k, 11-4-1 antibody; K^b, 20-8-4 antibody; negative (Neg.) control, 19/178, Lyt 2.2 antibody.

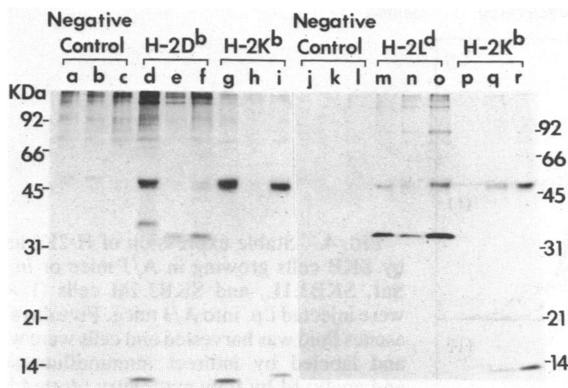

FIG. 2. Immunoprecipitations of H-2K^b antigen from SKB and L929-K^b cells. Nonidet P-40 extracts of [^{35}S]methionine-labeled cells were immunoprecipitated with mAb ascitic fluid and protein A-Sepharose and analyzed on 12.5% NaDodSO$_4$/PAGE gels. EL4 (lanes l, d, and g), L929 (lanes b, e, and h), L929-K^b (lanes c, f, and i), SaI (lanes j, m, and p), SKB3.1L (lanes k, n, and q), and SKB3.1M (lanes l, o, and r) were immunoprecipitated with PG14.1 (negative control mAb), 28-14-8 (D^b/L^d mAb), and 20-8-4 (K^b mAb).

SKB and L929-K^b cells (lanes i, q, and r). These polypeptides comigrate with H-2 heavy chain and β_2m of the EL-4 tumor, respectively. No polypeptides were immunoprecipitated by 20-8-4 from untransfected parental SaI or L929 cells (lanes h and p). The endogenously expressed L^d polypeptides of the parental and transfected SaI cells are immunoprecipitated by mAb 28-14-8 (lanes m, n, and o). These results demonstrate that the transfected SaI and L929 cells express a molecule that is serologically and biochemically indistinguishable from native H-2K^b antigen.

Expression of the H-2K^b Alloantigen on SaI Tumor Cells Does Not Alter the Cells' Tumorigenicity. To test the effect of H-2K^b antigen expression on the tumorigenicity of SaI cells, A/J mice were challenged with the parental tumor and five SKB clones. As shown in Fig. 3, eight of nine A/J mice injected i.p. with 1 × 10^5 SaI cells died within 40 days of tumor challenge. A/J mice challenged in parallel with four of five of the SKB clones (SKB3.1D, SKB3.1E, SKB3.1M, SKB3.1O) show survival kinetics similar to the parental SaI line. Eleven of 13 of the A/J mice challenged with the SKB3.1L clone, however, survive the tumor challenge. A/J mice challenged with 1 × 10^4 SKB3.1M or SKB3.1E cells show survival times similar to mice challenged with 1 × 10^5 cells (data not shown). B10.A (K^{k}D^{d}L^d) mice challenged with SaI, SKB3.1L, and SKB3.1M cells have survival times similar to those of A/J tumor-challenged mice (data not shown). Therefore, despite the expression of the H-2K^b alloantigen on the sarcoma cells, A/J and B10.A mice are incapable of rejecting most of the SKB clones. As shown in Table 2, allogeneic C57BL/6 (*H-2^b*) and C3H/HeJ (*H-2^k*) mice reject tumor challenges of 1 × 10^5 or 1 × 10^6 SaI cells or SKB cells.

Table 2. Survival times of mice challenged with SaI and SKB cells

Host	Inoculum size (cells)	Cells	Mice, no.	Survival, days
C57BL/6	1 × 10^5	SaI	5	>177
C3H/HeJ	1 × 10^5	SaI	5	>202
C57BL/6	1 × 10^6	SaI	4	>279
C57BL/6	1 × 10^5	SKB3.1M	5	>234

Mice were inoculated i.p. with the designated number of cells and followed for tumor incidence and survival. Survival shown indicates the time period for which they were followed.

To ascertain that A/J mice are capable of rejecting an H-2^b tumor, A/J mice were challenged i.p. with the *H-2^b* thymoma EL-4. Seven of seven A/J hosts are fully resistant to this tumor at an inoculum of 1 × 10^5 cells (survival time, >6 months).

Given the tumorigenicity of most of the SKB clones, tumor cells were examined for the *in vivo* stability of H-2K^b antigen expression. SKB3.1M (as a representative high tumorigenic clone) and SKB3.1L cells were inoculated i.p. into A/J mice. Tumor cells were removed from the peritoneal cavity 5 days later and, as assessed by indirect immunofluorescence, H-2K^b antigen was strongly expressed on both SKB3.1L and SKB3.1M cells (Fig. 4 *f* and *i*). Similar findings have been obtained for SKB3.1L and SKB3.1M cells grown in A/J mice for 2 weeks (data not shown). Therefore, the H-2K^b antigen is stably expressed on the SKB cells *in vivo*.

SaI and SKB Clones Are Not Susceptible to CTL-Mediated Lysis. Because SKB and SaI cells are equally tumorigenic in A/J and B10.A mice, they have been assessed for their susceptibility to H-2K^b-specific and H-2K^b-restricted virus-specific CTL. As shown in Fig. 5*A*, A/J anti-B10.A(5R) (anti-H-2K^b) CTL effectively lyse EL-4 and L929-K^b target cells but fail to lyse SKB, SaI, or L929 cells. The same transfectants were tested for their susceptibility to lysis by the H-2K^b-specific BM 11-41 CTL clone (Fig. 5*B*). EL4 and

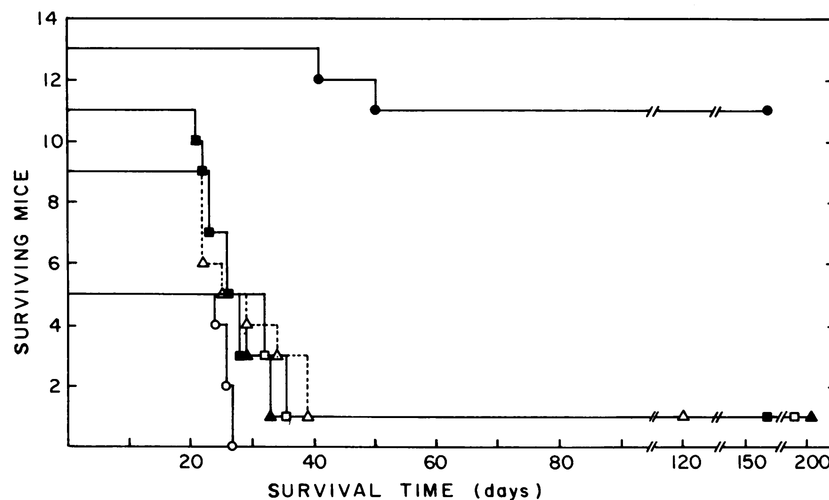

FIG. 3. Survival times of A/J mice challenged with SaI cells or SKB clones. Mice were inoculated i.p. with 1 × 10^5 SaI (△), SKB3.1D (□), SKB3.1E (○), SKB3.1L (●), SKB3.1M (■), or SKB3.1O (▲) cells and followed for survival. All mice showed large ascites tumors at the time of death.

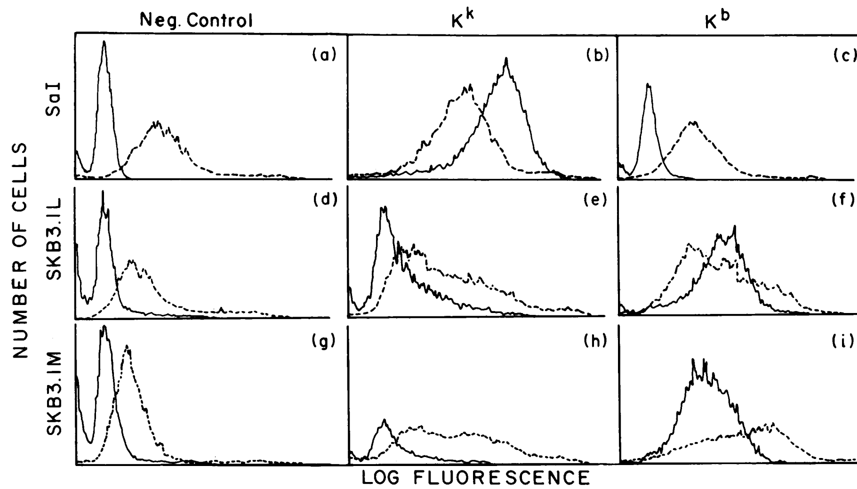

FIG. 4. Stable expression of H-2K^b antigen by SKB cells growing in A/J mice or *in vitro*. SaI, SKB3.1L, and SKB3.1M cells (1×10^5) were injected i.p. into A/J mice. Five days later, ascites fluid was harvested and cells were washed and labeled by indirect immunofluorescence and analyzed by flow cytometry (dotted lines). Each profile represents 5×10^3 cells from an individual mouse. Control *in vitro* cultured cells (solid lines) were labeled and analyzed under identical conditions.

L929-K^b cells are highly susceptible to lysis by this clone, whereas no lytic activity is seen against SKB3.1L and SKB3.1M cells. Similar results have been obtained with BM 10-38, another H-2K^b-specific CTL clone (data not shown). SaI cells are also not killed by allogeneic CTL effectors specific for H-2K^k and H-2D^d antigens (data not shown).

SaI and SKB cells were also tested as targets for H-2K^b-restricted LCMV-specific CTL. Primary LCMV-specific CTL produced in B10.A(5R) (K^{b}D^d) mice lysed LCMV-infected L929-K^b targets but not LCMV-infected SKB cells (Table 3).

Due to their failure to be lysed by specific CTL, SaI, SKB3.1L, and SKB3.1M cells were tested for susceptibility to LDCC. Concanavalin A (Con A)-induced C57BL/6 effector cells readily lysed Con A-treated EL4 and P815 target cells but did not lyse SaI or SKB cells (Table 4).

The failure of SaI and SKB cells to be lysed by bulk cultured and cloned alloreactive CTL, H-2K^b-restricted LCMV-specific CTL, and LDCC suggests that these tumor cells are refractory to cell-mediated lysis.

DISCUSSION

The parental SaI tumor described in the present studies is strain specific in that it does not grow in allogeneic hosts. Other reported SaI sublines are also strain specific. Only one early study describes the growth of SaI in certain allogeneic tumor recipients. The absence of strain specificity in these studies was only seen, however, when mice were challenged with SaI cells grown in donors who had been pretreated with large quantities of tumor or normal tissue extracts (40). Therefore, under normal tumor challenge conditions, the SaI tumor behaves as expected for an allograft and follows the established rules of transplantation. It would therefore be expected that SaI tumor cells expressing an allogeneic MHC class I antigen should be recognized by the host's immune system as an allograft. This recognition should result in rejection of the tumor by an immunocompetent host. However, four of five of the H-2K^b antigen-expressing SaI clones are not rejected by A/J or B10.A mice, suggesting that the expression of an allogeneic MHC class I antigen on this tumor is insufficient for immune-mediated rejection.

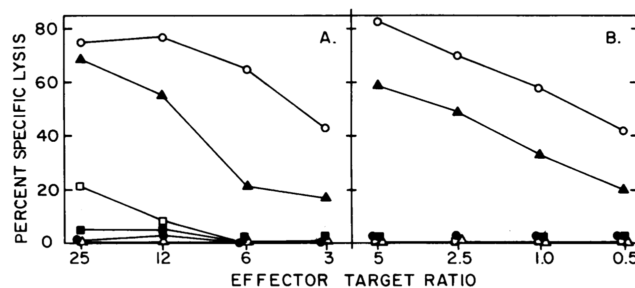

FIG. 5. Susceptibility of SKB and L929-K^b cells to lysis by H-2K^b-specific CTL. Cells were tested for their ability to serve as targets for A/J anti-B10.A(5R) CTL (*A*) and H-2K^b-specific CTL clone BM11-41 (*B*) in a 4-hr ^{51}Cr release assay. Targets are EL4 (○), L929 (□), L929-K^b (▲), SaI (△), SKB3.1L (●), and SKB3.1M (■).

Tumor growth may be due to an inherent insensitivity of the sarcoma cells to immune-mediated cytolytic mechanisms. SaI and SKB cells are resistant to *in vitro* CTL-mediated lysis (Fig. 5). However, the parental tumor and those transfectant clones tested are readily rejected by C57BL/6 (*H-2^b*) and C3H (*H-2^k*) mice. Studies by others using various strains of mice have demonstrated that immunity to the SaI tumor is T-cell mediated (41) and is probably directed against allogeneic MHC antigens (42).

Lack of rejection of the SKB clones may be due to the expression of a structurally altered or unstably expressed H-2K^b antigen that is not immunologically recognized as an allogeneic class I molecule. However, the H-2K^b antigen expressed on the transfected cells exhibits the following properties. (*i*) It reacts strongly with mAb directed against epitopes of the first and second domains of the H-2K^b molecule (Fig. 1, Table 1). (*ii*) It is 45 kDa in size and is associated with β_2m (Fig. 2). (*iii*) It shows the same lateral diffusion coefficient as other MHC class I antigens and as the endogenous H-2D^b antigen (M. Edidin, personal communication). (*iv*) It is stably and uniformly expressed *in vitro* and *in vivo* (Figs. 1 and 4). (*v*) When expressed by L929 cells, it serves as a target/restriction element for anti-H-2K^b and class I-restricted virus-specific CTL (Fig. 5, Table 3). Therefore, the *H-2K^b* gene used for the transfections encodes a func-

Table 3. Cytotoxic activity of B10.A(5R) LCMV immune spleen cells on LCMV-infected target cells

	% specific ^{51}Cr release		
E:T ratio	L929-Kb	L929	SKB3.1L
25:1	29	5	0
12:1	33	7	0
6:1	25	4	0

Mice were infected with 5×10^3 plaque-forming units of LCMV-UBC i.p. and immune spleens were harvested 8 days later. Target cells (1×10^6) were infected with 0.3 plaque-forming unit per cell 48 hr prior to use and infection was confirmed by indirect immunofluorescence. Results shown are for a 6-hr ^{51}Cr release assay. E:T, effector-to-target.

Table 4. LDCC of Con A C57BL/6 splenic effector cells on SKB target cells

E:T ratio	% specific ^{51}Cr release				
	EL4	P815	SaI	SKB3.1L	SKB3.1M
10:1	47	52	0	0	1
5:1	42	43	0	0	0
2.5:1	30	36	0	0	0

C57BL/6 spleen cells were cultured for 72 hr with 4 μg of Con A per ml. Spleen cells were washed three times and incubated in parallel with target cells with or without Con A at 5 μg/ml. Target lysis in the absence of Con A did not exceed 6%. E:T, effector-to-target.

tionally active molecule that is structurally identical (at the limits of our resolution) to other H-2K^b antigens.

That SKB clones may be tumorigenic in A/J hosts because A/J mice fail to respond to H-2K^b alloantigen is unlikely because A/J mice reject the EL-4 tumor and can make anti-H-2K^b CTL (Fig. 5*A*).

Studies by others (41) suggest that a primary inoculum of SaI cells is lethal in A/J mice, not because this tumor is poorly immunogenic but because a primary immune response is inadequate to control tumor growth. In the present experiments, it cannot be excluded that the immune system of A/J and B10.A mice is overwhelmed by the SKB clones; however, it is unlikely for the following reasons. If the immune response was being overloaded, then a reduction in tumor inoculum size should be accompanied by a decrease in tumor frequency. This situation is not true, however, for SaI or SKB cells. Tumor inocula of 1×10^3, 1×10^4, or 1×10^5 SaI or SKB3.1M cells are equally tumorigenic (data not shown). Therefore, over a 100× range of tumor cells tumorigenicity is unrelated to inoculum size. Furthermore, since C57BL/6 and C3H mice are capable of rejecting challenges of 1×10^5 and 1×10^6 SaI and/or SKB cells, SKB cells expressing clearly detectable allodeterminants should presumably be rejected by A/J and B10.A mice.

One of the five SKB clones (SKB3.1L) shows reduced tumorigenicity in A/J and B10.A mice. It is unclear why this clone is phenotypically less tumorigenic than SaI or the other SKB clones.

The results reported here differ significantly from other recent studies demonstrating the sufficiency of a MHC class I antigen for tumor rejection. Adenovirus type 12-transformed MHC antigen-negative tumor cells are highly tumorigenic. Transfection with an *H-2L^d* gene results in a reduction or loss of tumorigenicity (20). Murine line 1 lung carcinoma cells are very low expressers of MHC class I molecules and are highly tumorigenic. Transfection of line 1 cells with the *H-2D^p* gene results predominantly in clones that have lost their tumorigenicity in allogeneic hosts (22). One clone, however, retained tumorigenicity despite the expression of the *H-2D^p* gene. Therefore, for some tumor cells, there is a direct correlation between the expression of MHC class I antigens and tumorigenicity, but for other tumor cells the expression of class I molecules alone is apparently insufficient to reduce tumorigenicity.

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