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Immunology

Reversal of the metastatic phenotype in Lewis lung carcinoma cells after transfection with syngeneic *H-2K^b* gene

(tumor metastasis/gene transfer/histocompatibility antigens/immunotherapy)

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ABSTRACT **High metastatic clones of the murine 3LL carcinoma express greatly reduced levels of H-2K^b major histocompatibility complex class I antigens, while low metastatic clones of the same tumor express high levels of H-2K^b. Induced expression of this antigen after transfection with the *H-2K^b* gene resulted in conversion of a metastatic to a non- or low-metastatic phenotype. Unlike the parental cells, transfected cells are potent inducers of H-2K^b-restricted syngeneic cytotoxic lymphocytes that kill the K^b-positive clones and cross-react with parental nontransfected cells. Preimmunization of mice with K^b-positive transfectants conferred protection against metastatic spread of malignant cells. Moreover, immunotherapy of metastasis was achieved by immunization with the H-2K^b-transfected cells of animals already carrying a growing local tumor of the parental cells.**

The major histocompatibility complex class I gene products are cell-surface proteins essential for the immune recognition of, and consequently the elimination by, cytotoxic T cells (CTLs) of neoplastic and virus-infected cells (1, 2). Reduced levels of expression of H-2 class I proteins were observed in spontaneous, chemically induced, and virally transformed murine tumors (3–8) and in human small-cell lung carcinomas (9), neuroblastomas (10), teratocarcinoma (11), eccrine protocarcinomas (12), cervical carcinomas (13), and Burkitt lymphoma lines (14). In many murine tumors, the lack of H-2 expression determines reduced immunogenicity and high tumorigenicity. Thus, in adenovirus type 12-transformed cells, suppression of H-2K, H-2D, and H-2L was correlated with tumorigenicity, whereas restoration of H-2K^b (and less so of H-2L^d) expression inhibited tumor growth (15). In AKR lymphomas and RadLV-induced thymomas (16), suppression of H-2K^k was causally related to tumorigenicity and transfection of *H-2K^k* genes increased the immunogenic properties of these cells, consequently suppressing growth *in vivo* (17).

Studying the metastatic, as distinct from tumorigenic, properties of neoplastic cells, we described (5) the T10 sarcoma system of heterozygous (H-2^b × H-2^k)F$_1$ origin in which metastatic and nonmetastatic clones completely lack cell-surface expression of both *H-2K^b* and *H-2K^k* genes. The metastatic properties of T10 clones were positively correlated with the expression of H-2D^k antigens (18). We showed (19) that transfection by either *H-2K^b* or *H-2K^k*, or both genes, conferred immunogenic properties on the various T10 clones and resulted in abrogation of the metastatic competence of the highly metastatic clone.

The Lewis lung carcinoma (3LL) tumor that originated spontaneously in a C57BL H-2^b mouse lacks cell-surface expression of H-2K^b antigen (3). Screening a large number of newly generated single cell clones of 3LL, we showed that an inverse relationship existed between the metastatic phenotype of a clone and the relative expression of H-2K^b and H-2D^b glycoproteins: the lower the H-2K/H-2D ratio, the higher the metastatic competence of the clone (4). The low metastatic H-2K^b-positive clones were significantly more immunogenic, both in syngeneic and in allogeneic hosts, compared to high metastatic H-2K-negative cells (20). It appeared that the putative cell-surface tumor antigen in the 3LL tumor could be recognized by CTLs only in association with *H-2K^b* gene products, but not when associated with *H-2D^b* (20). *In vitro* treatment by γ-interferon transiently activated the expression of the *H-2K^b* gene in the metastatic clones (21). When such cells were injected intraveneously into mice a significant decrease in their metastatic competence was observed (21–23). γ-Interferon is known to have many different functions in addition to increasing the expression of major histocompatibility complex antigens. Accordingly, it was decided to test the hypothesis that the level of expression of the H-2K^b antigen is the critical factor in determining whether or not metastasis takes place. Transfection experiments were then carried out. *H-2K^b* genes were transfected into cells of the highly metastatic H-2K^b-negative clone D122. Cells of this highly metastatic clone reverted to low- or nonmetastatic phenotypes after transfection with the *H-2K^b* gene. This was correlated with the acquisition of H-2K-restricted immunogenic properties.

MATERIALS AND METHODS

Mice. C57BL/6 and C3H/eb mice were obtained from The Jackson Laboratory.

Cell Cultures. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (4). *H-2K^b*-transfected clones were selected in gentamycin (400 μg/ml) and maintained in gentamycin (200 μg/ml).

Plasmids and Probes. An H-2K^b plasmid, a 10.5-kilobase (kb) *Eco*RI genomic fragment cloned into pBR328, was used for transfection (24). A 30-mer H-2K^b-specific oligonucleotide, CACAGCTCCAGTGACTATTGCAGCTCCAAG, an H-2K-specific probe p1955 (25), and a general H-2 class I probe H8Pst8 (24) were used for DNA and RNA hybridizations.

DNA Transfection. Twenty micrograms of H-2K^b plasmid and 2 μg of pSV$_2$neo, which contains the gene encoding gentamycin resistance (26), were transfected into 5 × 10^5 D122 cells by the calcium phosphate technique (19, 27).

RNA Blot Analysis. Total RNAs were isolated as described by Chirgwin *et al.* (28), and RNAs were electrophoresed on 1% agarose/2.2 M formaldehyde gels, blotted onto nitrocellulose, and hybridized in 50% (vol/vol) formamide/10% (wt/vol) dextran sulfate at 42°C. Plasmid inserts were labeled by nick-translation and oligonucleotides were end-labeled by

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Abbreviation: CTL, cytotoxic T lymphocyte.

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T4 polynucleotide kinase, as described by Maniatis *et al.* (29).

Detection of Cell-Surface Antigens. Monoclonal antibodies 28-13-3, 20-8-4 (anti-H-2K^b), and 28-14-8 (anti-H-2D^b) (30) were purified from ascites fluids on protein A-Sepharose. Monoclonal antibodies K7-309, K9-136, K10-56, Y-25, and H-100-5-28 (anti-H-2K^b) were concentrated by ammonium sulfate from tissue cultured hybridoma cells (31). For microfluorimetry assays, single cell suspensions were incubated with monoclonal antibodies at 0°C for 1 hr, washed, and stained with fluorescein-conjugated goat anti-mouse immunoglobulin. The stained cells were analyzed in the Becton Dickinson FACS II analyzer. For direct radioimmunoassays, purified antibodies were labeled with Na^{125}I by chloramine T. Cell suspensions (5×10^5 cells per tube) were incubated with 1 μg of labeled antibodies in triplicate in bovine serum albumin-coated tubes for 2 hr at 0°C. Cells were washed four or five times with phosphate-buffered saline/0.5% bovine serum albumin/0.02% azide, and monitored in a γ-scintillation counter.

Tumor Development and Metastatic Assays. *Tumor growth and spontaneous metastasis.* Ten mice in each experimental group were inoculated intrafootpad (i.f.p.) with 10^5 cells per mouse. Local tumor growth was determined by measuring the footpad diameter with calipers. The tumor-bearing leg was amputated as soon as the tumor reached a diameter of 8–10 mm and the mice were killed 21 days post-amputation. The lungs were assessed for metastatic load.

Experimental metastases. Ten mice were inoculated via the tail vein with 5×10^5 tumor cells. Three to 4 weeks later, the mice were sacrificed, the lungs were weighed, and the number of nodules was determined after fixation in Bouin's solution.

Immunizations. Mice were immunized by two protocols: (*i*) preimmunization by three weekly i.p. injections of 2×10^6 cells per mouse. The cells were irradiated with 5000 R and treated with mitomycin C (80 μg/ml). On day 9 or 10 after the third booster injection, the mice were challenged i.f.p. or i.v., or spleens were removed for *in vitro* assays. (*ii*) Preimmunization by one injection of 10^6 living cells into the pinna of the ear. Spleens were removed on day 9 or 10.

***In Vitro* Cytotoxicity.** Spleen cells from preimmunized mice were restimulated *in vitro* on monolayers of irradiated tumor cells (5000 R) treated with mitomycin C (80 μg/ml) and cultured in RPMI medium/10% fetal calf serum for 5 days. Viable lymphocytes were separated by lymphoprep centrifugation (Cedarlane, ON, Canada) and admixed at different ratios with 5000 [^{75}Se]selenomethionine-labeled target cells in u-shaped microtiter wells. The plates were incubated for 16 hr at 37°C. Cultures were terminated by centrifugation at 300 $\times$ *g* for 10 min at 4°C and 100-μl supernatants were assayed in a γ-scintillation counter. Percentage specific lysis was calculated as follows:

$$\frac{\text{experimental } ^{75}\text{Se release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100. \quad [1]$$

Maximal release was determined by solubilization of target cells in 1% Nonidet P-40.

RESULTS

Transfected D122 Cells Express H-2K^b Antigens. The entire *H-2K^b* gene, including 5′ and 3′ flanking regions, in pBR328 (24) was transfected into D122 tumor cells. Cotransfection with pSV$_2$neo (26) and selection in gentamycin yielded 30 clones positive for cell-surface H-2K. Parallel transfection by pSV$_2$neo alone did not change H-2K expression on D122 cells. Ten of the H-2K transfectants were recloned by limiting dilution and DNA, RNA, and protein were analyzed by specific DNA probes and monoclonal antibodies. DNA blot analysis with an H-2K-specific probe, 1955 (25), showed insertion of tandem multiple copies of the gene at one or more integration sites. RNAs from the same clones were probed with an H-2K^b-specific oligonucleotide (Fig. 1*A*), with the H-2K-specific probe 1955 (Fig. 1*B*), and with a general class I probe, H8Pst8 (Fig. 1*C*; ref. 24), and compared to RNA from the highly metastatic H-2K^b nonexpressor D122 clone and to RNA from low-metastatic H-2K^b expressor clone A9. Clone D122 does not express H-2K-related mRNA, while clone A9 shows a 1.8-kb H-2K^b transcript. Nine of the 10 transfected clones showed very high steady-state levels of H-2K^b mRNA. Two different mRNA species, 1.8 and 2 kb, were transcribed from the transfected genes, probably by using alternative splicing sites for exon VIII (32), while the A9 expressor cells show only the shorter form. Although two protein products, differing in the C terminus of the *H-2K^b* gene, have been described (33), no evidence exists that the two forms of the molecule are functionally different. To

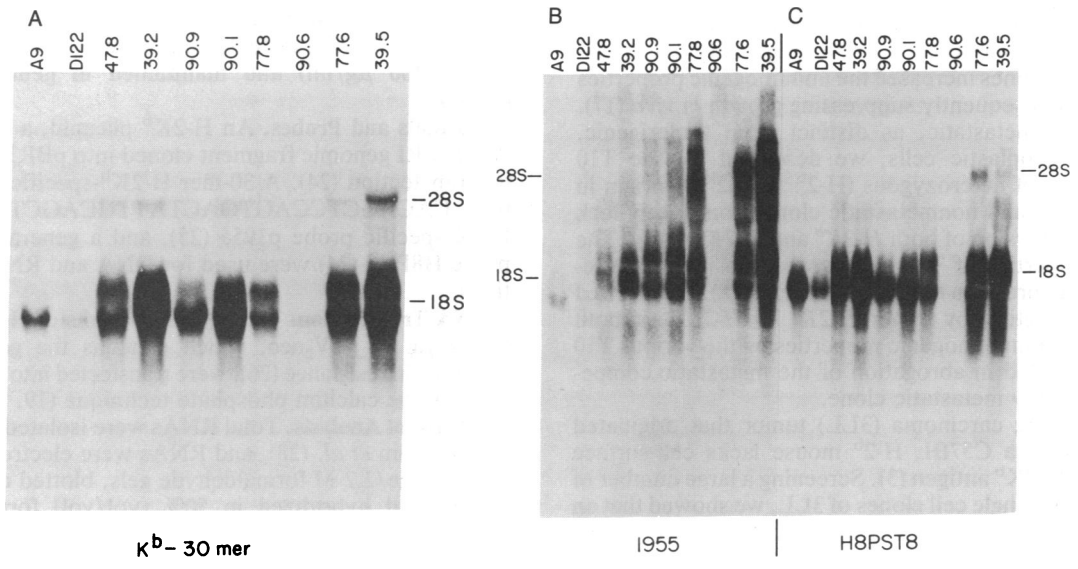

FIG. 1. Steady-state levels of H-2 mRNA in nonmetastatic A9, metastatic D122, and K^b-transfected D122 clones. (*A*) Oligonucleotide probe. (*B*) H-2K-specific probe 1955. (*C*) H-2 probe H8Pst8. After a 36-hr hybridization, blots were washed in 0.1% SSC/0.1% NaDodSO$_4$ (SSC = 0.15 M NaCl/0.015 M sodium citrate) at 65°C and exposed overnight.

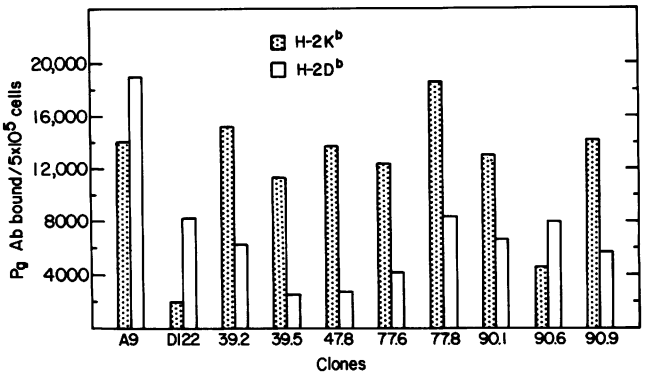

FIG. 2. H-2 phenotype of A9, D122, and K^b-transfected D122 clones. Direct RIA was performed as described.

evaluate the levels of cell-surface expression of H-2K^b antigens, transfected clones were screened by flow cytometry and by a direct radioimmunoassay with anti-H-2K^b antibodies. Antibodies 28-13-3, 20-8-4 (30), K7-309, K9-136, K10-56, Y-25, and H-100-5-28, which recognize different epitopes of the H-2K^b molecule (31), showed positive staining of clone A9 and of the K^b-transfected D122 cells. A representative radioimmunoassay (Fig. 2) shows that high levels of H-2K^b were expressed on the transfectants, excluding clone 90.6, which expressed relatively low levels of the transfected gene. Cell-surface expression of the *H-2D^b* gene in the transfectants was similar or lower than that of the parental D122 cells. Although H-2K mRNA levels are much higher in the transfectants than in the expressor clone A9 (Fig. 1), cell-surface expression of the H-2K^b molecules does not exceed that of A9 cells (Fig. 2). Whether this is due to limited amounts of β_2-microglobulin synthesized by the cells and necessary for insertion into the cell membrane (34) or to posttranscriptional regulatory events is not yet clear.

Tumorigenicity and Metastatic Properties. Tumorigenicity and metastatic properties of the eight H-2K^b-transfected clones were tested in syngeneic C57BL/6J mice. Most transfected clones were tumorigenic at 10^5 cells per mouse, and clone 39.2 showed the lowest tumorigenicity, manifesting tumor growth in only 5 of 10 mice at this inoculum (Table 1). Growth rates varied among the clones: while clones 90.6, 39.5, and 77.6 grew at rates similar to those of A9 and D122, others such as 90.9, 90.1, 77.8, and 39.2 showed retarded growth (data not shown).

Table 1 summarizes the generation of metastases after i.f.p. inoculation and after i.v. inoculation of the K^b transfectants. Most clones showed a nonmetastatic or a low-metastatic phenotype; clone 90.6, which expresses low levels of the H-2K^b protein, was moderately metastatic. The suppression of the metastatic spread occurred both when cells were inoculated i.f.p. and i.v. (Table 1). Twelve D122 clones transfected by neomycin alone were also tested and were found to remain highly metastatic (35). When recipient mice were sublethally irradiated (450 rad; 1 rad = 0.01 Gy) to inactivate partially host immune reactivity and then were inoculated i.v. with the *H-2K*-transfected cells of clones 77.8 and 90.1, metastases were generated. These findings suggested that the host's immune system is involved in abrogating metastasis formation by *H-2K^b*-transfected cells.

H-2K^b Transfectants Are Potent Inducers of and Are Susceptible Targets for Anti-Tumor CTLs. To test whether the suppression of metastases results from the generation of anti-tumor CTLs, syngeneic C57BL/6J mice were preimmunized by the two protocols described, and restimulated spleen cells were tested for cytotoxic activity against different target cells. A representative assay is shown in Fig. 3 (*A* and *B*). A single-dose immunization by parental D122 cells did not lead to the generation of effective CTLs (Fig. 3*B*). In contrast, a single immunization by 77.8 cells resulted in generation of a high frequency of anti-tumor CTLs. These CTLs lysed K^b transfectants efficiently and cross-reacted with the parental D122 cells. After immunization by three doses of the parental D122 cells, 45% of either parental D122 cells or K^b transfectant 77.8 were killed at high effector/target ratio (Fig. 3*A*). CTLs generated after immunization with three doses of 77.8 cells could kill 80% of the parental D122 cells at a ratio of 100:1. These CTLs were tumor specific and did not lyse unrelated K^b-positive cells such as EL4 lymphoma (data not shown). It thus appears that the CTL generated by the *H-2K^b*-transfected cells recognized tumor-associated antigens associated with *H-2K^b* gene products.

The low immunogenicity and high tumorigenicity of the metastatic clones such as D122 can also be demonstrated by the ability of these cells to grow as primary tumors in allogeneic recipients. In fact, 3LL cells can form tumors in any tested mouse strain (36). Our previous studies showed that the high metastatic K^b-negative D122 cells grew progressively in allogeneic recipients such as C3H/eb and BALB/c mice, while the low metastatic K^b-positive A9 cells were rejected by allogeneic mice (20). Inoculation of *K^b*-

Table 1. Metastatic phenotypes of H-2K^b-transfected D122 clones in syngeneic C57BL/6 mice

Clone	Major histocompatibility complex expression, pg per 5 × 10^5 cells		Growth of primary tumor	Spontaneous metastasis (i.f.p.)		Experimental metastasis (i.v.)	
	K^b	D^b		Average lung weight, mg	Average no. of nodules	Average lung weight, mg	Average no. of nodules
A9	14,100 ± 1457	19,333 ± 864	10/10	170 ± 40	2 ± 1	172 ± 15	2 ± 1
D122	1,964 ± 380	8,181 ± 572	10/10	623 ± 362	58 ± 27	487 ± 297	48 ± 26
			K^b-transfected D122 cells				
77.8	18,511 ± 92	8,339 ± 406	9/9	194 ± 17	0	156 ± 10	0
39.2	15,313 ± 635	6,323 ± 429	5/10	194 ± 12	0	210 ± 32	8 ± 8
39.5	11,366 ± 807	2,480 ± 128	10/10	170 ± 14	0	250 ± 32	3 ± 3
47.8	13,664 ± 1050	2,732 ± 203	8/9	166 ± 19	5 ± 6	239 ± 31	2 ± 2
90.9	14,096 ± 1020	5,598 ± 545	10/10	271 ± 81	13 ± 7	203 ± 35	7 ± 2
90.1	13,005 ± 1433	6,613 ± 500	7/10	278 ± 102	15 ± 12	164 ± 17	2 ± 2
77.6	12,422 ± 124	4,163 ± 607	9/9	275 ± 103	16 ± 8	267 ± 40	20 ± 12
90.6	4,600 ± 207	7,929 ± 970	9/9	337 ± 64	32 ± 6	367 ± 257	25 ± 11
			Irradiated mice				
D122			—	—	—	832 ± 128	100
77.8			—	—	—	242 ± 128	45 ± 5
90.1			—	—	—	282 ± 139	49 ± 48

Each group consisted of 9 or 10 mice.

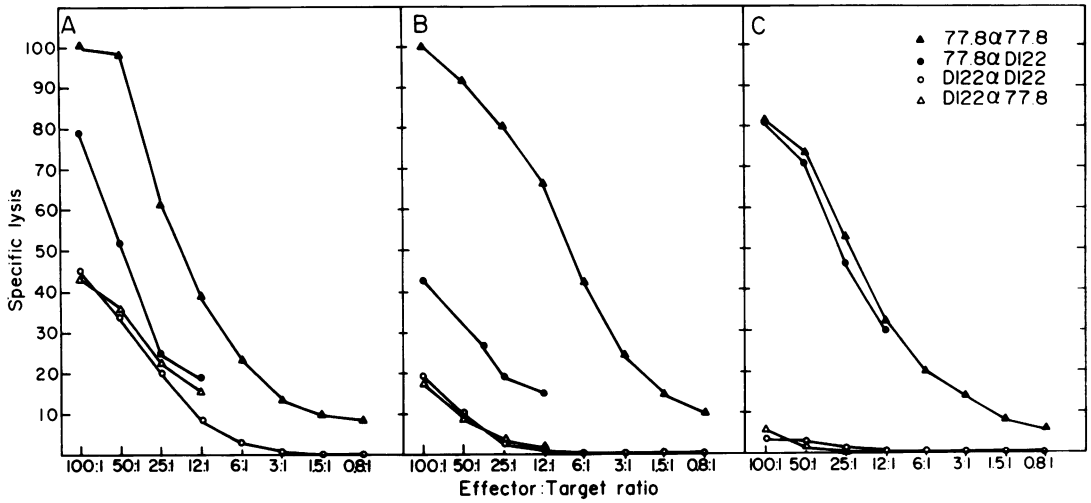

FIG. 3. *In vitro* lytic activity of CTLs elicited by clones D122 and 77.8. (*A*) Three i.p. injections in C57BL. (*B*) One pinnal injection in C57BL. (*C*) One pinnal injection in C3H/eb. [^{75}Se]selenomethionine-labeled target cells (D122 or 77.8) reacted in a 16-hr assay with increasing numbers of effector cells.

transfected D122 clones into C3H/eb or BALB/c mice resulted in tumor growth to 4–5 mm, followed by acute rejection of the growing tumor (data not shown). CTLs from C3H/eb mice immunized once in the ear by transfectant 77.8 could lyse both the K^b-positive 77.8 cells and the parental D122 cells (Fig. 3*C*) *in vitro*, while spleen cells from C3H/eb mice immunized by parental D122 cells did not elicit any CTL activity (Fig. 3*C*). The fact that D122 cells that express the products of the *H-2D^b* gene, allogeneic to the C3H/eb (*H-2K^k*, *H-2D^k*) host, do not elicit an immune response, while K^b-expressing D122 cells are effective immunizers, again stresses the differential functions of the H-2K^b antigen versus the H-2D^b antigen in the 3LL system.

These results indicate that transfection of *H-2K^b* genes into nonimmunogenic D122 cells is necessary and sufficient to convert them to nonmetastatic phenotypes, while turning them into potent immunizers capable of inducing the generation of a high level of CTLs that lyse most efficiently cells of K^b-positive clones and cross-react with the parental D122 cells.

H-2K^b-Positive Clones Can Immunize Syngeneic Animals Against Metastatic Growth *in Vivo*. We next tested whether the effective CTL demonstrated *in vitro* is correlated with syngeneic immune reactivity *in vivo*. C57BL/6J mice were preimmunized three times i.p. by irradiated D122, 77.8, or 90.1 cells, then challenged by 5×10^5 cells i.v. or by 10^5 cells i.f.p. In Table 2 we show that preimmunization by K^b transfectants 77.8 and 90.1 not only eliminated residual metastases otherwise formed by cells of the respective low-metastatic clones, but also reduced dramatically the metastases formed by the parental D122 cells after an i.v. challenge. An average of only 1 metastatic nodule was found after preimmunization with clone 77.8 and 7 nodules were found after preimmunization with clone 90.1, whereas nonimmunized animals generated 83 nodules or animals preimmunized by the parental D122 cells generated 37 nodules. When the preimmunized animals were challenged i.f.p., the K^b-positive clones were completely rejected, while the local growth of D122 cells and of A9 cells was significantly retarded (data not shown). Spontaneous postamputation metastases of D122 were also reduced compared to nonimmunized mice or to mice immunized by D122 cells. These results indicate that the increased immunogenicity achieved by K^b transfection may act to reduce the load of lung metastases formed by the parental cells.

To study the possibility that the antimetastatic immunity can act in preventing metastasis formation in animals already carrying a local parental tumor, groups of mice were inoculated i.f.p. by 2×10^5 D122 cells. Starting at day 8 after inoculation, when palpable tumors could be detected, groups of mice were immunized one to seven times i.p. by irradiated and mitomycin-treated cells (10^6 cells per mouse) of clones D122, 77.8, or 90.1 at weekly intervals. Unlike the preimmunization protocol described above, immunizations given after initiation of the local growth did not change the growth rate of the primary tumors. Recipient mouse legs were amputated according to our standard protocol, and the mice were sacrificed 30 days later, after the nonimmunized control

Table 2. *In vivo* immunogenic properties of D122- and *H-2K^b*-transfected cells

Immunization	Average lung weight, mg (average no. of metastatic nodules)			
	Control	77.8	90.1	D122
		Intravenous challenge		
77.8	274 ± 50 (4)	158 ± 13 (0)	170 ± 24 (0)	164 ± 23 (0)
90.1	281 ± 83 (12)	194 ± 31 (0)	223 ± 33 (0)	197 ± 19 (2)
D122	973 ± 303 (83)	207 ± 33 (1)	295 ± 99 (7)	478 ± 482 (37)
		i.f.p. challenge		
77.8	171 ± 9 (0)	*	*	*
90.1	288 ± 170 (8)	*	*	*
D122	672 ± 468 (50)	396 ± 246 (25)	311 ± 90 (15)	519 ± 375 (60)
A9	167 ± 17 (0)	170 ± 21 (0)	163 ± 18 (0)	174 ± 19 (0)

Immunized C57BL/6J mice (three times, i.p.) were challenged i.v. (5×10^5 cells per mouse) or i.f.p. (10^5 cells per mouse). Weights are given in mg ± SD. Ten mice per group.
*Rejection of primary tumors.

group and the group receiving one immunization with D122 cells had died with heavy loads of lung metastases. As described in Fig. 4, a single immunization with 77.8 cells dramatically reduced lung metastases. In groups receiving five to seven immunizations, most of the mice (50/57) were completely metastasis free. Immunization by clone 90.1 was slightly less efficient, two to five immunizations reduced by a factor of 3–4 the average metastatic load, and after seven immunizations 27/39 mice were metastasis free. The smallest effect was observed in groups immunized by the parental D122 cells. A single immunization did not reduce the metastatic spread of D122 cells. Three to six immunizations reduced by a factor of ≈2 the average metastatic load, and only after seven immunizations was a reduction by a factor of ≈3 observed. In conclusion, the immunogenic effect evoked by K^b-positive transfectants could efficiently prevent the metastatic growth of an established parental tumor.

DISCUSSION

The data presented here show that transfection of an *H-2K*^{b} gene into nonexpressor highly metastatic D122 cells abolished the metastatic capacity of these cells (Table 1). It thus appears that the *H-2K*^{b}-transfected cells acquired H-2K-restricted immunogenic competence, which was not sufficient to arrest local growth in normal animals but was potent in preventing the metastatic growth of disseminating cells. When H-2K^b transfectants were inoculated into preimmunized recipients, complete rejection took place (Table 2).

It seems, therefore, that the putative tumor-associated antigen of the 3LL elicits effective T-cell immunity only when associated with H-2K^b antigen, but not when associated with H-2D^b. Interestingly, preimmunization by K^b-positive cells decreased dramatically the metastatic spread of the parental D122 tumor (Table 2). In accordance, we demonstrated that CTLs evoked by K^b D122 cells could kill *in vitro* not only the K^b-positive clones but also the parental D122 cells (Fig. 3). The very low density of H-2K^b on D122 cells does not confer immunogenic competence but seems sufficient to make the cells susceptible to H-2K^b-restricted CTLs. A similar cross-immunity was reported by Hui *et al.* (17), who observed that preimmunization by *K*^{k}-transfected AKR leukemia K36-16 protected AKR mice against a subsequent challenge by nontransfected K36.16 cells. Also, Tanaka *et al.* (15) showed that *K*^{b}-transfected adenovirus type 12-transformed cells afforded partial protection *in vivo* against tumors of H-2-negative cells. In contrast, in the T10 sarcoma system, such cross-reactivity *in vitro* was not observed. CTLs induced by *K*^{b} or *K*^{k}-transfected IE7 or IC9 cells did not kill the parental lines, most probably because the parental T10 cells did not manifest any cell-surface H-2K expression (19). We further demonstrated that the immunity induced by irradiated and mitomycin-treated *K*^{b}-transfected D122 cells is also effective in the prevention of metastatic spread from an established parental tumor. Since increasing numbers of human tumors have been found to express very reduced levels of class I antigens (9–14), the prospect of antimetastatic immunotherapy by cells from the primary tumor manipulated to express class I major histocompatibility complex deserves investigation.

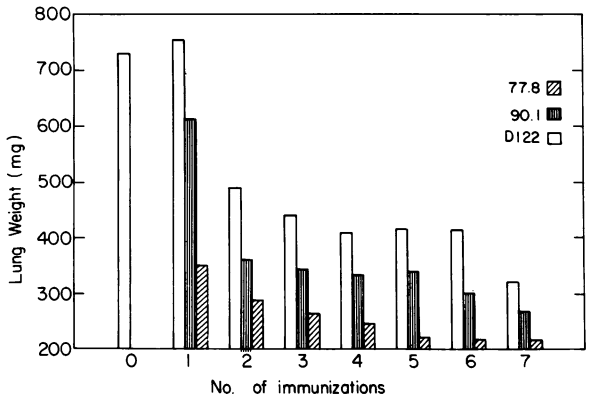

FIG. 4. Abrogation of the metastatic spread of D122 tumor cells as a function of the number of immunizations with K^b transfectants and parental D122.

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