nus of mature glucoamylase. Because both the primary signal peptidase cleveage and the secondary trypsin-like processing occur efficiently for pre-pro-glucoamylase, it may be possible to use the glucoamylase signal sequence to direct secretion of other foreign proteins from yeast.

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RESEARCH ARTICLE

Reversal of Oncogenesis by the Expression of a Major Histocompatibility Complex Class I Gene

Kenichi Tanaka, Kurt J. Isselbacher, George Khoury, Gilbert Jay

The immune system is involved not only in defense against infections but also against "spontaneously derived" aberrant cells (*1*). This latter immune function appears to be essential for the removal of autonomous cell variants that presumably arise frequently in multicellular organisms (*2*).

The development of malignant tumors, therefore, represents not only neoplastic transformation but the failure of host resistance to eliminate certain abnormal cells. Transformation of a cell is insufficient to ensure its escape from immune surveillance. Cells transformed in culture very often do not induce tumors when transplanted back into immunocompetent syngeneic hosts (*3*). It is those properties of certain tumor cells allowing them to resist immune recognition that are ultimately responsible for their tumorigenicity (*4*).

The major histocompatibility complex class I (H-2) antigens (designated K, D, and L in mice) are indispensable for the presentation of cells bearing "foreign" antigens to the cytotoxic T lymphocytes (*5*). The finding that certain malignant tumors, including teratocarcinomas (*6*), eccrine porocarcinomas (*7*), and cervical carcinomas (*8*), have markedly reduced or nondetectable levels of class I antigens at the cell surface (in contrast to their normal cellular counterparts) suggests a possible mechanism for their escape from immune surveillance. In support of this hypothesis is the recent finding that cells transformed by the highly oncogenic strain of human adenovirus (Ad12), in contrast to the nononcogenic strain (Ad5), also express reduced levels of class I antigens on their surfaces (*9, 10*).

This observation with Ad12 provides an experimental system for demonstrating that the absent or reduced expression of class I antigens is directly responsible for oncogenicity. We now show that transfection of a functional class I gene into a highly tumorigenic Ad12-transformed cell line that expresses no detectable class I surface antigens resulted in its complete loss of oncogenicity. This finding indicates one possible mechanism for the escape of certain tumors from immune surveillance and suggests future therapeutic approaches for the reversal of certain malignancies.

Expression of genes encoding class I antigens in Ad12-transformed mouse cell lines. Since it was not clear whether the suppression of class I antigens by Ad12 occurred only in rat cells and since at least one other study did not substantiate this finding (*11*), we sought to confirm and extend the observation. Two Ad12-transformed cell lines, designated C57AT1 and C3AT1 (*12*), established by transformation of embryonic cell cultures derived from C57BL/6 and C3H mouse strains, respectively, were selected.

Polyadenylated [poly(A)$^+$] RNA was extracted from these two cell lines and was compared to a preparation from nontransformed BALB/c3T3 cells by Northern blot analysis (Fig. 1). To determine the relative amounts of RNA in the three samples, the RNA blot was first hybridized with a ^{32}P-labeled genomic actin probe (Fig. 1, panel A). All three cell lines expressed comparable levels of an RNA transcript (~20*S*) characteristic of actin messenger RNA. Subsequent hybridization of the same RNA

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blot with the Ad12 early region 1A (E1A) genomic probe revealed the expression of the 12*S* and 13*S* RNA species (as well as a larger precursor component) in both C57AT1 and C3AT1 cells, but not in BALB/c3T3 cells (Fig. 1, panel B). An ~23*S* RNA species (and a precursor) hybridized to the early region 1B (E1B) genomic probe in the two Ad12-transformed cell lines but not in BALB/c3T3 cells (Fig. 1, panel C). The expression of E1A and E1B functions in both C57AT1 and C3AT1 cells demonstrated that the cell lines are stably transformed by Ad12.

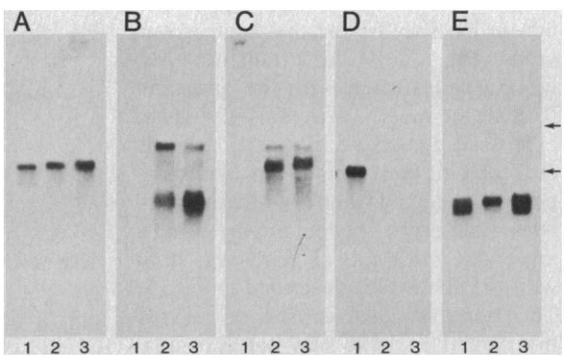
The expression of class I genes in these cells was examined with a complementary DNA (cDNA) probe derived from a segment of the L gene that detects all of the class I transcripts that accumulate in the cell (Fig. 1, panel D). An 18*S* species, characteristic of class I transcripts, was readily detected by this probe in RNA from BALB/c3T3 cells; no hybridization was observed with C57AT1 RNA, and a very low amount of hybridization was seen with C3AT1 RNA. Since the amount of RNA in all three cell lines was comparable (see Fig. 1, panel A), class I gene expression was totally suppressed in C57AT1 cells and reduced more than 20-fold in C3AT1 cells.

Because expression of class I antigens on the cell surface requires that the antigens physically associate with β_2-microglobulin (*13*), attempts to modulate the transcription of class I genes in Ad12-transformed cells depend on the β_2-microglobulin gene being active. The levels of transcription of the β_2-microglobulin gene were comparable among the three cell lines (Fig. 1, panel E). Differences between cell lines were observed in the broadness of the bands in the Northern analysis. This suggests that while both potential β_2-microglobulin polyadenylation sites appear to be utilized in BALB/c3T3 and C3AT1 cells, only the downstream recognition signal may be utilized in C57AT1 cells. Since there is no evidence that the two RNA species have different translational efficiencies (*14*), there is no reason to suspect that the expression of β_2-microglobulin is perturbed in either Ad12-transformed cell line.

The effect of class I antigen expression on tumorigenicity. The tumorigenicity of both transformed cell lines was tested in syngeneic hosts. Complete tumor response was observed when 5×10^5 C57AT1 cells were injected into C57BL/6 mice, and a comparable response was also found when 5×10^5 C3AT1 cells were injected into C3H/HeJ mice. As class I molecules are the major transplantation antigens responsible for graft rejection, the Ad12-transformed cells should also be accepted in allogeneic immunizations. The C57AT1 cells (H-2^b haplotype) induced a virtually 100 percent tumor response in BALB/c mice (H-2^d haplotype) at a dose of 5×10^6 cells. The higher cell dose required for allogeneic immunization (BALB/c mice), as compared to syngeneic immunization (C57BL/6 mice), might be explained by the involvement of minor histocompatibility antigens or the presence of residual class I antigens in C57AT1 cells.

The fact that Ad12-transformed cells are highly tumorigenic even across histocompatibility barriers provided the opportunity to introduce, by DNA-mediated gene transfer, a cloned class I gene whose product was serologically and biochemically distinguishable from those

Abstract. *The classical transplantation antigens (the major histocompatibility complex class I antigens) play a key role in host defense against cells expressing foreign antigens. Several naturally occurring tumors and virally transformed cells show an overall suppression of these surface antigens. Since the class I molecules are required in the presentation of neoantigens on tumor cells to the cytotoxic T lymphocytes, their absence from the cell surface may lead to the escape of these tumors from immunosurveillance. To test this possibility, a functional class I gene was transfected into human adenovirus 12–transformed mouse cells that do not express detectable levels of class I antigens; the transformants were tested for expression of the transfected gene and for changes in oncogenicity. The expression of a single class I gene, introduced by DNA-mediated gene transfer into highly tumorigenic adenovirus 12–transformed cells, was sufficient to abrogate the oncogenicity of these cells. This finding has important implications for the regulation of the malignant phenotype in certain tumors and for the potential modulation of oncogenicity through derepression of the endogenous class I genes.*



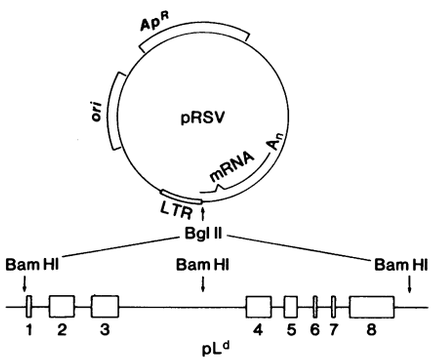


Fig. 1 (left). Characterization of messenger RNA expressed in Ad12-transformed mouse cells by Northern blot analysis. Poly(A)$^+$ RNA obtained from BALB/c3T3 cells (lanes 1), C57AT1 cells (lanes 2), and C3AT1 cells (lanes 3), was fractionated in a 1.0 percent agarose gel, in the presence of formaldehyde, as described (*28*). The RNA was then transferred from the gel to a nitrocellulose membrane and the resulting RNA blot was hybridized successively to one of five ^{32}P-labeled DNA probes at 45°C in 50 percent formamide, 5× standard saline citrate, 10 percent dextran sulfate, and 5× Denhardt's solution. (Panel A) Nick-translation probe derived from a genomic clone of human actin (*29*); (panel B) nick-translation probe derived from a genomic clone of Ad12 E1A gene (*30*); (panel C) nick-translation probe derived from a genomic clone of Ad12 E1B gene (*30*); (panel D) single-stranded primer-extension probe derived from an L^q cDNA clone that spans the region encoding amino acid 237 to a position 239 base pairs downstream from the termination codon in the region of all class I genes (*28*); (panel E) nick-translation probe derived from a cDNA clone of mouse β_2-microglobulin (*14*). The top arrow indicates the position of 28*S* ribosomal RNA and the bottom arrow that of 18*S* ribosomal RNA. Fig. 2 (right). The construction of a recombinant plasmid that carries the complete L^d gene immediately downstream of the Rous sarcoma virus LTR. In addition to the plasmid origin of replication and the gene for ampicillin resistance (Ap^R), the pRSV vector contains the RSV LTR (*15*) which is followed by the SV40 t-antigen splice site and polyadenylation site (A_n) (*16*). A single Bgl II recognition sequence has been placed at the 3′ side of the LTR for the insertion of a partial Bam HI fragment of ~4.5 kilobases which contains the entire L^d gene.

encoded by the endogenous loci which are suppressed in these transformed cells.

To avoid the possibility that the Ad12 function, which is directly or indirectly responsible for repression of the endogenous class I loci, might also suppress the exogenously introduced class I gene, the cloned L^d gene was modified by the replacement of its 5′ flanking sequences with the Rous sarcoma virus long terminal repeat (RSV LTR) (*15*). The RSV LTR contains both a transcriptional enhancer and a promoter (*16*) (Fig. 2). In this chimeric construction, all of the exons of the L^d gene were retained, including that encoding the polyadenylation site (*17*). Since the 5′ cap site of the L^d gene has not been identified, we must presume that initiation of transcription will be directed by the RSV promoter. In either case, the size of the L^d transcript would not be detectably different.

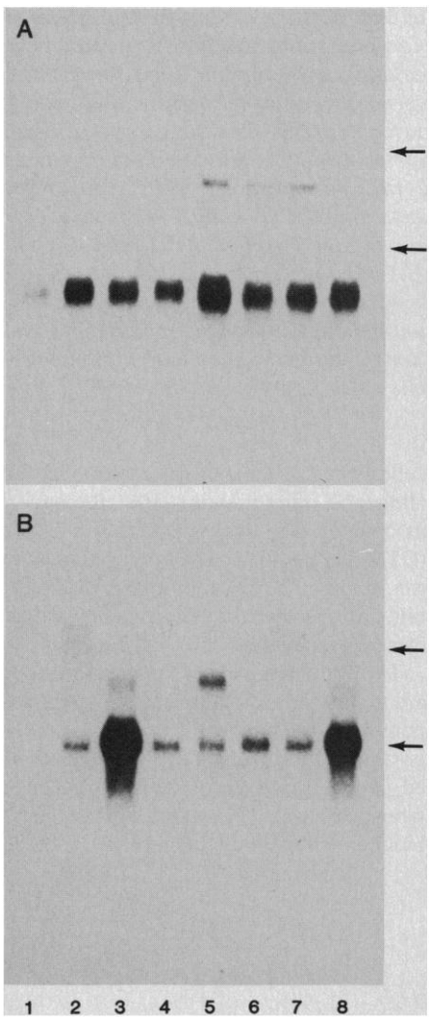


Fig. 3. Characterization of messenger RNA expressed in individual clones transfected with either the native L^d gene or the RSV L^d gene. Poly(A)$^+$ RNA obtained from the parental tumor C57AT1 (lanes 1), from four individual clones with the transfected L^d gene, designated L8 (lanes 2), L9 (lanes 3), L12 (lanes 4), and L14 (lanes 5), and from three individual clones with the transfected RSV L^d gene, designated R2 (lanes 6), R3 (lanes 7), and R4 (lanes 8). The RNA was fractionated in a 1.0 percent agarose gel and transferred to a nitrocellulose membrane. The resulting RNA blot was hybridized with a nick-translation probe derived from a genomic clone of the Ad12 E1A gene (*30*) (A), denatured, and rehybridized with an end-labeled synthetic oligonucleotide probe that is specific to the L gene (*25*) (B). The top arrow in each panel indicates the position of 28*S* ribosomal RNA and the bottom arrow that of 18*S* ribosomal RNA.

The cloned L^d and the chimeric RSV L^d genes were separately introduced into C57AT1 cells by cotransfection with pRSVNEO (a derivative of pBR322 carrying the neomycin resistance gene under the control of the RSV LTR) in the presence of G418 selection (*18*). Individual neomycin-resistant clones were first tested for the stable integration of the appropriate L^d gene. Poly(A)$^+$ RNA was obtained from two sets of transformants, one group transfected with the L^d gene (designated clones L8, L9, L12, and L14) and the other with the RSV L^d gene (designated clones R2, R3, and R4). These RNA's and RNA from the original C57AT1 tumor (as control) were then analyzed by Northern blot hybridization (Fig. 3). The Ad12 E1A genomic probe was used for hybridization to establish the maintenance of the transformed phenotype and that the relative amount of RNA was comparable among the samples (Fig. 3A). All seven transformants expressed similar levels of both Ad12 E1A and E1B transcripts. For reasons that are unclear, the relative abundance of the E1A transcript in each of the transformants was higher than in the parental C57AT1 tumor.

To determine whether the transfected class I gene was expressed in the stable transformants, a synthetic oligonucleotide probe specific for the L gene was used (Fig. 3B). All transformants, whether transfected with the authentic L^d gene or the chimeric RSV L^d gene, expressed detectable but varying levels of L-specific transcripts. No hybridization was detected when a synthetic oligonucleotide probe specific for the K gene (an endogenous class I gene) was used with the RNA blot described in Fig. 3. This suggests that expression of the transfected L^d gene was not accompanied by derepression of the endogenous K^b gene. Thus, the Ad12 function cannot act, either directly or indirectly, in *trans* to repress the expression of the transfected L^d gene. This is true whether it is under the control of its own regulatory elements or under the control of a heterologous regulatory sequence such as the RSV LTR. There was no detectable difference in efficiency between the homologous and heterologous control signals. The class I gene expression in clone L9 (transfected with the authentic L^d gene) and clone R4 (transfected with the chimeric RSV L^d gene) are much higher than those observed in other transformants, but are comparable to levels observed in BALB/c3T3 cells.

The cell-surface localization of the L^d antigen in both L9 and R4 was confirmed by indirect immunofluorescence staining of live cells with an L^d-specific monoclonal antibody, designated 30.5.7 (Fig. 4). While this antibody did not detectably stain the parental C57AT1 cells (panel A), both L9 (panel B) and R4 (panel C) showed intense fluorescence at the cell surface comparable to that observed with BALB/c3T3 cells. Incubation of the same set of cells with a K^b-specific monoclonal antibody, designated 34.1.2 (*19*), resulted in no detectable cell surface fluorescence. This finding also confirms that the endogenous class I genes remained suppressed while the transfected L^d gene was efficiently expressed.

Since the C57AT1 cells are highly tumorigenic when transplanted into BALB/c mice and express high levels of

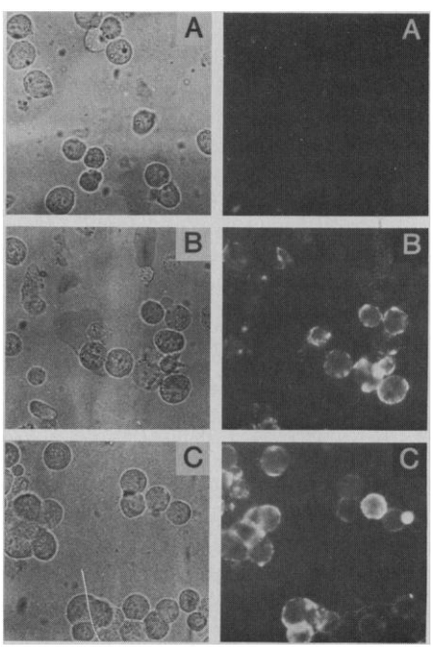


Fig. 4. Immunofluorescent staining of the L^d antigen on the surfaces of individual clones. C57AT1 cells (panels A), L9 cells containing the transfected L^d gene (panels B), and R4 cells containing the transfected RSV L^d gene (panels C) were trypsinized, incubated with a mouse monoclonal antibody 30.5.7 directed against the L^d antigen (*19*), further treated with rhodamine-conjugated rabbit antibody to mouse immunoglobulin G, and visualized with a fluorescence microscope (*31*). Each field of cells was photographed both with bright-field optics (left) and with fluorescence optics (right).

the corresponding class I surface antigen when transfected with the BALB/c-derived L^d gene, it was possible to determine whether oncogenicity induced by Ad12 could be reversed by the expression of a class I antigen that is syngeneic with the host. Two high-producer clones (L9 and R4) and two low-producer clones (L8 and R3), together with the parental C57AT1 cell line, were tested for tumorigenicity in BALB/c mice (Fig. 5). While the low-producer clones L8 and R3 were as tumorigenic as C57AT1, high-producer clones L9 and R4 were greatly reduced in tumorigenicity. At a cell dose of 3×10^7, 50 percent of the animals died (TD_{50}) at about 28 days for the control and low-producer cells, and at about 55 days for the high-producer cells (Fig. 5C). When the number of cells in the inoculum was reduced to 1×10^7 per animal, the TD_{50} for the control and low-producers was found at 37 days, and that for the high-producers was delayed to >60 days (Fig. 5B). When the cell number was further decreased to 5×10^6 per animal the TD_{50} for the control and low-producers was about 40 days (as an average); there were virtually no deaths observed for the high-producer clones (Fig. 5A). Regardless of the cell dose, the clones expressing high levels of the transfected L^d gene (clones L9 and R4) were much less tumorigenic in BALB/c mice ($H\text{-}2^d$ haplotype) than the parental cells.

A determination of the average tumor size in each group of animals also led to this conclusion (Fig. 6). At a dose of 5×10^6 cells, C57AT1 and R3 cells induced gradual increases in tumor size that ultimately led to the death of virtually all animals. In contrast, R4 cells induced tumors which rapidly regressed; all of the animals ultimately appeared "cured" and healthy (Fig. 6A). When the cell dose was increased to 1×10^7, the induction of tumors by C57AT1 and R3 was more rapid. At this dose, R4 cells induced a bimodal response reflecting rapid regression of tumors in some, but not all, animals (Fig. 6B). This bimodal response induced by R4 cells was detectable even at a dose as high as 3×10^7 cells (Fig. 6C). These findings are consistent with the interpretation that tumor growth outpaces the rejection response when the tumor cell dose is sufficiently high. In other words, when the challenging dose is not too high, the host succeeds in completely eradicating all tumor cells. With increasing tumor doses, fewer and fewer individuals are capable of completely resisting the challenge. Nevertheless, the host response is indicated by the increase in the survival time of all animals within the group.

As further demonstration that the L^d antigen on the surface of the transfected clones is recognized by the immune system, both L9 and R4 cells, along with C57AT1, were transplanted into C57BL/6 ($H\text{-}2^b$ haplotype) mice. Since the L^d gene was derived from BALB/c ($H\text{-}2^d$ haplotype) mice, this immunization was expected to induce an allogeneic reaction which would lead to the rejection of both L9 and R4 cells, but not the control C57AT1 cells. The results obtained are in agreement with this expectation (Fig. 5, D to F) and confirm the immune recognition of the novel class I antigen in these cells.

Discussion. The effectiveness of the host's immune system in recognizing and destroying tumor cells depends not only on the magnitude of the immune response but also on the capacity of tumor cells to evade destruction. The lack of recognition by the immune system will suffice to allow tumor cells to escape surveillance, a phenomenon that will permit tumors to grow, metastasize, and kill.

Since neoantigens on tumor cells are recognized only in context with MHC class I antigens (*5*), failure to react to a foreign determinant can simply be the result of a deficiency in expression of endogenous class I antigens. There is evidence to suggest that some benign tumors express class I antigens while malignant tumors of the same tissue derivation do not. This correlation is based on clinical observations with, in particular, teratocarcinomas (*6*), eccrine porocarcinomas (*7*), and cervical carcinomas (*8*).

In confirmation of a previous observation with rat cells (*10*), we have observed that mouse embryo cells transformed by Ad12 have greatly reduced or undetectable levels of expression of class I antigens. Transfection of a cloned class I gene containing its own regulatory elements resulted in efficient expression under conditions where the endogenous genes are repressed. We have demonstrated that the Ad12 function directly or indirectly responsible for the suppression of class I gene expression cannot act solely in *trans*. Furthermore, introduction of a functional class I gene into the highly tumorigenic Ad12-transformed cell line resulted in complete loss of oncogenicity at certain tumor cell doses. However, as the challenging dose increased, tumor growth outpaced the mobilization of the rejection response despite the expression of class I antigens. Although survival was significantly prolonged due to immunological intervention, virtually all animals eventually succumbed to a high dose of tumor cells.

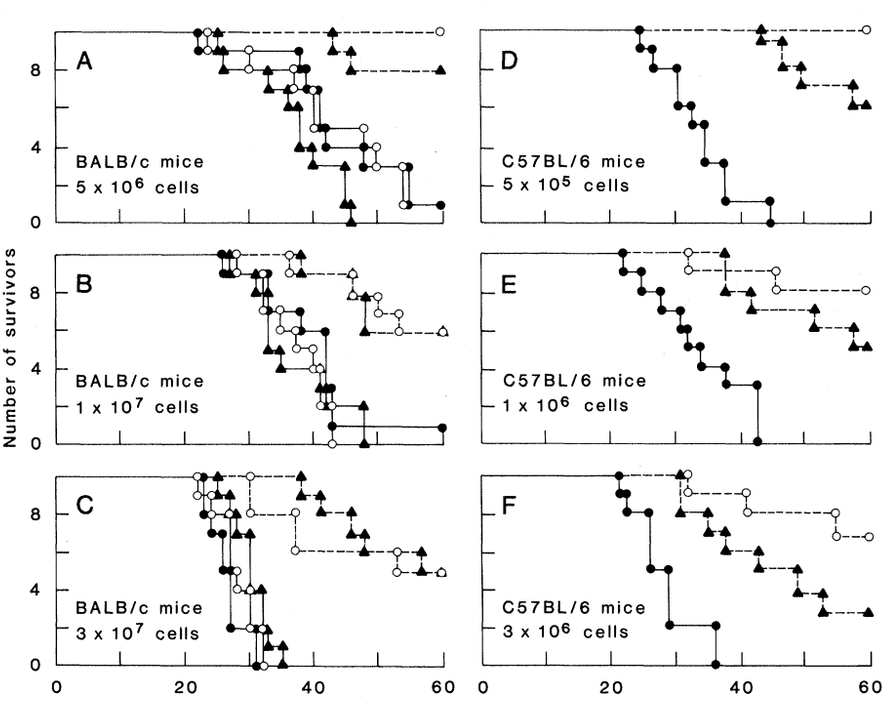


Fig. 5. Survival rates of mice injected with varying doses of tumor cells. Groups of ten mice, either BALB/c or C57BL/6, were each given a subcutaneous injection in the thigh with a fixed dose of cells in phosphate-buffered saline. The mice were 6 weeks old at the start of the experiments. Cell lines used were C57AT1 (●—●), L8 (▲—▲), L9 (▲- -▲), R3 (○—○), and R4 (○- -○).

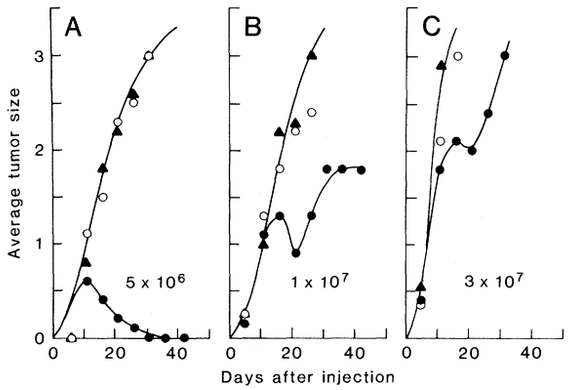


Fig. 6. Average tumor size in mice injected with varying doses of tumor cells. Groups of ten BALB/c mice were each given a subcutaneous injection in the thigh with a fixed dose of cells in phosphate-buffered saline. At regular intervals thereafter, the diameter of the tumor mass in each animal was measured. The cell lines plotted were C57AT1 (▲), R3 (○), and R4 (●).

This latter observation supports the rationale for removing or killing portions of the cells either by surgery or radiation treatments (or both), in conjunction with any attempt at immunotherapy for the treatment of cancer.

While the suppression of class I gene expression has been demonstrated with transformed cells in culture, a similar phenomenon may also be achieved by immunoselection in syngeneic hosts. Although cells transformed by SV40 in culture continue to express class I antigens, their serial passage in a syngeneic host results in enhancement of tumorigenicity and concomitant suppression of class I gene expression (*20*). It seems likely that the low level of expression of class I antigens in several natural tumors (*7, 8*) is the result of similar immunoselection processes which results in their escape from surveillance.

Suppression of MHC class I genes is not a universal characteristic of malignant tumors. In fact, in certain instances, stimulation of these genes has been observed. Lymphomas in AKR (H-2^k haplotype) mice, for example, show a significant increase in total class I antigens on the cell surface (*21*). This change appears to be a reflection of a marked increase in the expression of the D^k antigen (*22*). The level of the K^k antigen, however, remains essentially unchanged, although an increase or decrease has been observed in a small number of individual lymphomas (*22*). This observation is not restricted to AKR lymphomas but is also true in radiation leukemia virus (RadLV)–induced thymomas (*23*). One such AKR leukemia cell line (designated K36.16), which expresses a reduced level of K^k but a fourfold increase in D^k, was used to determine whether the introduction of a K^k gene by transfection has an effect on tumorigenicity (*24*). While the results were consistent with the transfected K^k gene in the atypical K36.16 cell line inducing tumor rejection, the implications of that experiment are not entirely clear because of the variability in the expression of the K^k antigen and the marked increase in the D^k antigen in AKR lymphomas in general.

Suppression of class I genes has been observed in a variety of carcinomas (*6–8*), which are derived from epithelial cell types. The generality of this observation, however, awaits the analysis of more carcinomas with well-defined DNA probes (*25*). In the case of Ad12-induced tumors, the expression of all class I genes is coordinately suppressed. The addition of a single class I gene was able to significantly reduce the oncogenicity of the Ad12-transformed cell line. This result, therefore, does not support the argument that different class I antigens play different yet essential roles in the immune response.

One approach to cancer therapy may be to modulate the expression of endogenous class I genes in certain tumor cells. Success in such attempts will depend upon defining the factors that regulate the expression of this family of MHC genes in both normal and transformed cells (*26*). A potential group of immunomodulators which may play such a role are the interferons. The α, β, and γ interferons induce a marked increase in the surface expression of class I antigens (*27*). While the molecular mechanism for this phenomenon has not been defined, our results suggest that it may underlie the efficacy of interferon in the treatment of certain malignancies.

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