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The effect of class II gene transfection on the tumourigenicity of the H-2K-negative mouse leukaemia cell line K36.16

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SUMMARY

There is much evidence to suggest that potentially immunogenic tumour cells can escape cytolytic immune destruction by loss of class I antigen expression. Many tumours are allele-specific class I negative and, in murine systems, reconstitution of class I expression by gene transfection leads to an increase in tumour immunogenicity. In many systems where mice have rejected class I transfected tumour cells they are also immune to a subsequent challenge with the untransfected parent tumour. In this study we have examined the effect of stable class II antigen expression (induced by gene transfection) on a class I loss mutant (H-2K^k negative) murine cell line, K36.16. We show that H-2E^k expression is more effective at increasing tumour immunogenicity than the reconstitution of H-2K^k expression in these cells. This suggests that the induction of class II antigen expression on tumour cells may provide an effective way of enhancing tumour-specific immune responses *in vivo*.

INTRODUCTION

It is now well established that CD4$^+$ T cells play a pivotal role in the immune response. That is, both antibody and cell-mediated immunity are dependent on initial recognition of antigen presented in the context of class II major histocompatibility (MHC) antigens to CD4$^+$ T (helper) cells. In contrast, cytotoxic T cells (CD8$^+$) have been shown to be restricted by MHC class I antigens. It has thus been proposed that tumour cells, which can evoke an immune response *in vivo* because of expression of either tumour antigens (TSTA) or viral antigens, can escape recognition and subsequent lysis by loss of expression of specific class I alleles. Many reports have documented the MHC class I loss phenotype in both rodent and human tumours.[1,2] We have shown that the highly tumourigenic leukaemic cell line K36.16 derived from the Gross leukaemia-susceptible AKR mouse does not express H-2K but becomes much less tumourigenic after transfection with and subsequent expression of the H-2K^k gene.[3] The surprising result obtained in these experiments was that mice which had been immunized with H-2K^k-transfected K36.16 cell lines (K36.K^k) were protected against a subsequent challenge with the unmodified K36.16 parent cell line. This implies that although H-2K-restricted cytolytic T cells may be important in the elimination of the K36.K^k tumour, other effector cell mechanisms must be involved.

In this study we show that the tumourigenicity of the K36.16 line can also be abolished if the K36.16 tumour expresses the class II gene product, H-2E^k. The defect in killing of K36.16 cells by AKR mice may not be solely at the level of H-2K^k restricted CTL killing but also in the *induction* of non-H-2K^k-restricted cytotoxic killer cells.

Abbreviations: CTL, cytotoxic T lymphocyte; Ii, invariant chain; K36.E^k, K36.16 cells transfected with Eα^k and Eβ^k genes (clone D2.5.2D6); K36.K^k, K36.16 cells transfected with the H-2K^k gene (clone C27.2.3); LN, lymph node; MHC, major histocompatibility complex; s.c., subcutaneous; TSTA, tumour-specific transplantation antigen.

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MATERIALS AND METHODS

Mice

AKR/J mice were obtained from the MRC breeding unit (Mill Hill, London, U.K.). Animals were maintained under clean, temperature-controlled and well-ventilated conditions on a step-wise light–dark cycle.

Tumour cell lines

The AKR-derived leukaemic cell line K36.16 has been described previously.[1] K36.16 does not react with a number of anti-H-2K^k monoclonal antibodies (mAb) and does not express H-2K^k-specific mRNA, as judged by S1 protection assay.[3] It does express relatively high levels of H-2D^k antigen and grows readily in AKR mice. K36.16 cells expressing the H-2K^k gene (C27.2.3) were obtained by transfection experiments described previously.[3] Tumour cells were maintained in culture in 'complete' RPMI-1640 medium containig 10% foetal calf serum (FCS), 4 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 U/ml streptomycin (Flow Laboratories Ltd, Rickmansworth, Herts, U.K).

DNA vector and DNA transfection procedures

The cosmid clones cosH-2^{k}18.1 (Eα^k) and cosH-2^{k}7.1 (Eβ^k)[4] were transfected into K36.16 cells by a modified calcium phosphate precipitation procedure.[5] Purified cosmid DNA from the two clones (5 μg of each) was mixed with 1 μg of purified pTCF,[6] which carries the aminoglycosyl-3′-phosphotransferase gene, and K36.16 cells were subsequently selected in medium containing 800 μg/ml Geneticin G418 (Gibco, Grand Island, NY). Transformed cells were harvested at Day 20 and cloned by limiting dilution in 96-well plates. Wells growing single colonies were harvested, expanded in complete RPMI-1640 containing 800 μg/ml G418 and tested for H-2E^k expression by immunofluorescence. The transfected cloned cell lines studied were D2.5.2D6, D2.5.A11 (both H-2E^k positive and G418 resistant) and D2.5.3A6 (H-2E^k negative and G418 resistant).

Radiobinding assays

Cells (5×10^5/well) were plated out in round-bottomed 96-well plates in a volume of 50 μl. Monoclonal antibody at the appropriate dilution (50 μl) was added to quadruplicate wells, the plates gently vortexed and left to incubate at room temperature for 1 hr. The cells were washed three times by centrifugation at 500 ***g*** for 1 min and resuspended in 100 μl of minimal essential medium containing 5% FCS. Bound antibody was detected by addition of 100 μl of ^{125}I-labelled sheep anti-mouse Ig (^{125}I-S anti-mIg) (50,000 c.p.m./100 μl). The cells were incubated for 30 min at room temperature and washed four times as above. After the last wash the cell pellets were dried at 37° and each plastic well containing a cell pellet cut from the plate and counted. Background binding using ^{125}I-S anti-mIg was subtracted. Monoclonal antibodies (obtained from ATCC, Rockville, MD) used were 11.4.1 (anti-H-2K^k), HB32 (anti-H-2E^k) and 100/27/55 (anti-H-2K^{k}D^k).

Fluorescence assays

Cells were incubated with mAb as above, washed three times and then 100 μl of FITC conjugated sheep anti-mIg (Nordic, Maidenhead, Berks, U.K.) added. After a 30 min incubation at room temperature the cells were washed three times, fixed in paraformaldehyde (0·1%) and assessed for fluorescence visually using an Olympus NHS microscope equipped with a mercury vapour lamp and epilluminator.

Tumour challenge experiments

Syngeneic (AKR) mice were injected with tumour cells subcutaneously (s.c.) in the base of the tail (for lymph node priming) or in the thigh region in a volume of 100 μl of medium. Tumour growth was monitored and recorded daily by the Biomedical Services staff. For secondary challenge experiments mice were left for 4 weeks before being rechallenged with untransfected K36.16 tumour cells (5×10^5 mouse).

Generation of CTL

The generation of CTL was a modification of previously established procedures.[7] AKR mice were immunized subcutaneously (SC) in the base of the tail with 10^6 viable tumour cells. Popliteal and inguinal lymph nodes were removed 4 days later and dissociated into a single cell suspension. Control lymph node (LN) cells were obtained by dissociating regional lymph nodes from normal unimmunized AKR mice. LN cells (2×10^6/ml) were cultured in 10 ml of 'complete' RPMI using a 25 cm^2 tissue culture flask in an upright position. After 3 or 5 days at 37° and 5% CO_2 cells were harvested and resuspended in a fixed volume of medium ready for use as effector cells in a 4 hr ^{51}Cr-release assay.

^{51}Cr-release assay

Tumour target cells were labelled with ^{51}Cr (sodium chromate, Amersham, Bucks, U.K.; no. CJS4) using approximately 200 μCi for 5×10^6 tumour cells. AKR splenic concanavalin A (Sigma, Poole, Dorset, U.K.) blasts were generated by 48 hr incubation at 2 μg/ml and labelled with ^{51}Cr as above. After incubating at 37° for 1–2 hr cells were washed three times and resuspended to 10^5 cells/ml. One-hundred microlitres of ^{51}Cr-labelled tumour cells were added to 100 μl of medium containing various numbers of effector cells (each in duplicate) in 96-well U-bottomed microtitre plates. The plates were then centrifuged at 200 ***g*** for 1 min prior to incubation for 4 hr at 37° in a humidified 5% CO_2 incubator. After 4 hr 100 μl cell-free supernatant were harvested from each well and the radioactivity determined by scintillation counting. Percentage specific lysis was calculated as follows:

$$100 \times \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})}.$$

Spontaneous release and maximum release were determined by incubating target cells in medium or in 1 M HCl, respectively.

RESULTS

Expression of H-2E^k on K36.16 cells

G418-resistant cells were tested for H-2E^k expression with anti-H-2E^k (HB32) using immunofluorescence and subsequently cloned by limiting dilution. The frequency of H-2E^k-expressing G418-resistant transfected cells (approximately 1 per 10^6) was relatively low compared to previous experience using H-2K^k and H-2K^b genes to transfect K36.16 cells.[5,8] Nevertheless a number of H-2E^k-positive clones were derived, of which two (2D6 and A11) were extensively studied. These cells have a stable low level expression of H-2E^k and were still H-2E^k-positive after more than 50 subcultures (Fig. 1).

Effect of H-2E^k-expression on tumourigenicity

The H-2E^k-transfected K36.16 clones A11 and 2D6 (K36.E^k) were immunogenic *in vivo*. As shown in Table 1, 90–95% of mice survived inoculation with A11 or 2D6 (5×10^5 cells, s.c.). Also, all mice surviving the initial inoculation were immune to a further challenge, 4 weeks later, of a highly tumourigenic dose (5×10^5 cells/mouse) of K36.16 cells. Normally such a high dose would give a large visible tumour within 10–14 days. In titration experiments >90% of AKR mice survived a challenge of 4×10^6 K36.E^k cells (A11 or 2D6). However, K36.K^k cells given at 4×10^6/mouse always gave 0% survival.

It is interesting to note that the K36.E^k cells gave rise to a solid tumour which was readily apparent 10–14 days post-inoculation and which later regressed, sometimes disappearing within 24–48 hr. This is in contrast to K36.16 cells, which do give a tumour apparent at about the same time period but which

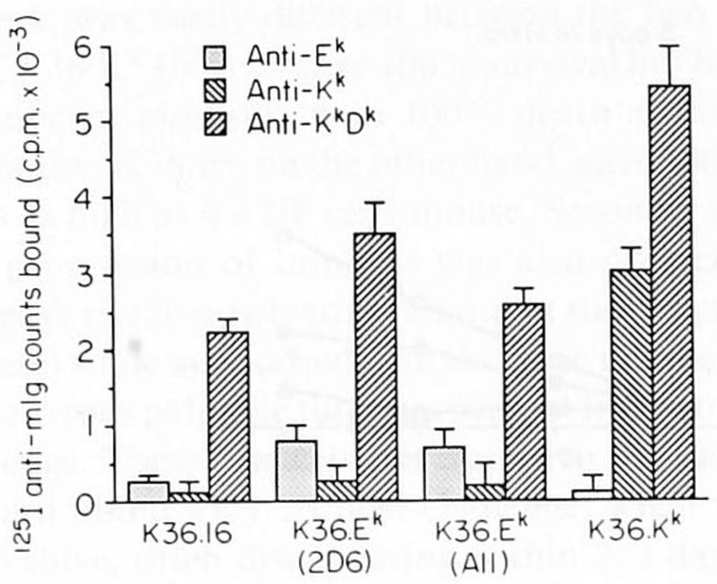

Figure 1. Expression of H-2E^k was tested regularly on cell lines 2D6 and A11 by a radiobinding assay using ^{125}I-sheep anti-mIg as a second layer. Counts bound by ^{125}I-sheep anti-mIg alone have been subtracted. The 2D6 and A11 cells had been passaged more than 50 times prior to the binding assay shown and had a stable but low expression since selection by cloning. Counts shown are mean ± SD.

inevitably progresses, and also to K36.K^k (H-2K^k-transfected K36.16 cells),[3] which does not give rise to a palpable tumour when given at a sub-lethal dose of 10^5/mouse or less. It has been noted that 17% of allogenic class I-transfected K36.16 cells (K36.K^b) can also give rise to a palpable tumour which regresses but the majority do not.[8]

Generation of tumour cell immunity

In vivo. After an initial challenge with K36.E^k cells a subsequent challenge at 4 weeks with the untransfected K36.16 parent cells did not lead to a tumour (Table 1). This phenomenon has been shown previously with both K36.K^k and K36.K^b cells.[3,8] However, when K36.E^k cells were given simultaneously with K36.16 cells a dose-dependent protection effect was seen (Table 2). This was apparent whether the class II-transfected cells were given at the same site or at a distant site. In contrast, K36.K^k cells gave no protection when given at the same doses (Table 2).

In vitro. An *in vitro* cytotoxicity assay was used to investigate induction of CTL after priming *in vivo* with K36.16, K36.K^k and K36.E^k. Lymph node cells were harvested 4 days post-priming and then cultured for various time periods before being tested for their ability to lyse tumour cells using a ^{51}Cr-release assay.

Table 1. Effect of H-2E^k gene transfection of tumourigenicity of K36.16 cells

Cells injected	G418 resistant	H-2E^k expression	No. of mice surviving*	No. of mice surviving after a secondary challenge with K36.16*
K36.16	—	—	0/10	N/A
D2.5.3A6	+	—	0/10	N/A
D2.5.2D6	+	+	14/15	14/14
D2.5.A11	+	+	9/10	9/9

*AKR mice were injected with 5×10^5 cells and mice surviving at 4 weeks were challenged with 5×10^5 K36.16 cells.

Table 2. Protection of lethal tumour induction by 10^5 K36.16 cells with co-immunization of K36.K^k and K36.E^k cells

No. cells injected	No. of mice surviving			
	Same site*		Distant site*	
	K36.K^k	K36.E^k	K36.K^k	K36.E^k
10^6	0/10	5/10	0/10	5/10
5×10^5	0/10	1/10	0/10	3/10
10^5	0/10	2/10	0/10	1/10
5×10^4	0/10	0/10	0/10	1/10

* Cells either mixed and injected together in the right flank (same site) or injected on the left flank with K36.16 cells (10^5) and then injected on the right flank with test cells (distant site). Mice injected with 10^5 K36.16 cells alone gave 0% survival. K36.E^k cells are the D2.5.2D6 line (see text).

Control LN cells from unimmunized mice always gave less than 5% lysis (data not shown). As can be seen, Fig. 2, minimal cytolytic activity was detectable upon priming with K36.16. However, both K36.E^k and K36.K^k cells induced significant CTL responses. Data are shown after 3 and 5 days *in vitro* culture. This pattern of killing was consistently observed in more than five separate experiments. Priming with either K36.K^k or K36.E^k cells generated tumour-specific CTL which could lyse all three targets (AKR Con A blasts were not lysed above control levels). This is consistent with the *in vivo* results (Table 1).

DISCUSSION

The MHC loss phenotype of many tumours has been well described.[9,10] This has led to the concept that tumour cells which display immunogenic epitopes may escape immune detection (and thus destruction) by lacking an appropriate restriction element.[11] Many studies have been performed to induce expression of MHC antigens on tumour cells by either somatic cell fusion of DNA-mediated gene transfer.[12] Becuase of their well-defined role in restricting cytotoxic T cells,[13] such studies have been done largely on MHC class I antigens, either syngeneic or allogeneic.[14] Although the methodology for transfecting class II genes is well established[15,16] it has only recently been used to study the effect on tumour cell growth *in vivo*.[17] The effect of invariant chain (Ii) expression on antigen presentation by class II-transfected tumour cells has also been studied[18] and it has been postulated that in the absence of Ii, TSTA presentation to T helper cells may be enhanced.[17] The innate expression of class II antigens by non-lymphoid malignant cells is a comparatively rare occurrence[19] and is associated with a better prognosis in some tumour systems.[20] In this study we have induced expression of an MHC class II antigen (H-2E^k negative) in an MHC class I loss mutant cell line (K36.16, H-2K^k negative) and studied the effects on tumourigencity. Conceptually, the induced (aberrant) expression of class II antigens on tumour cells could lead to a direct (and efficient) presentation of tumour

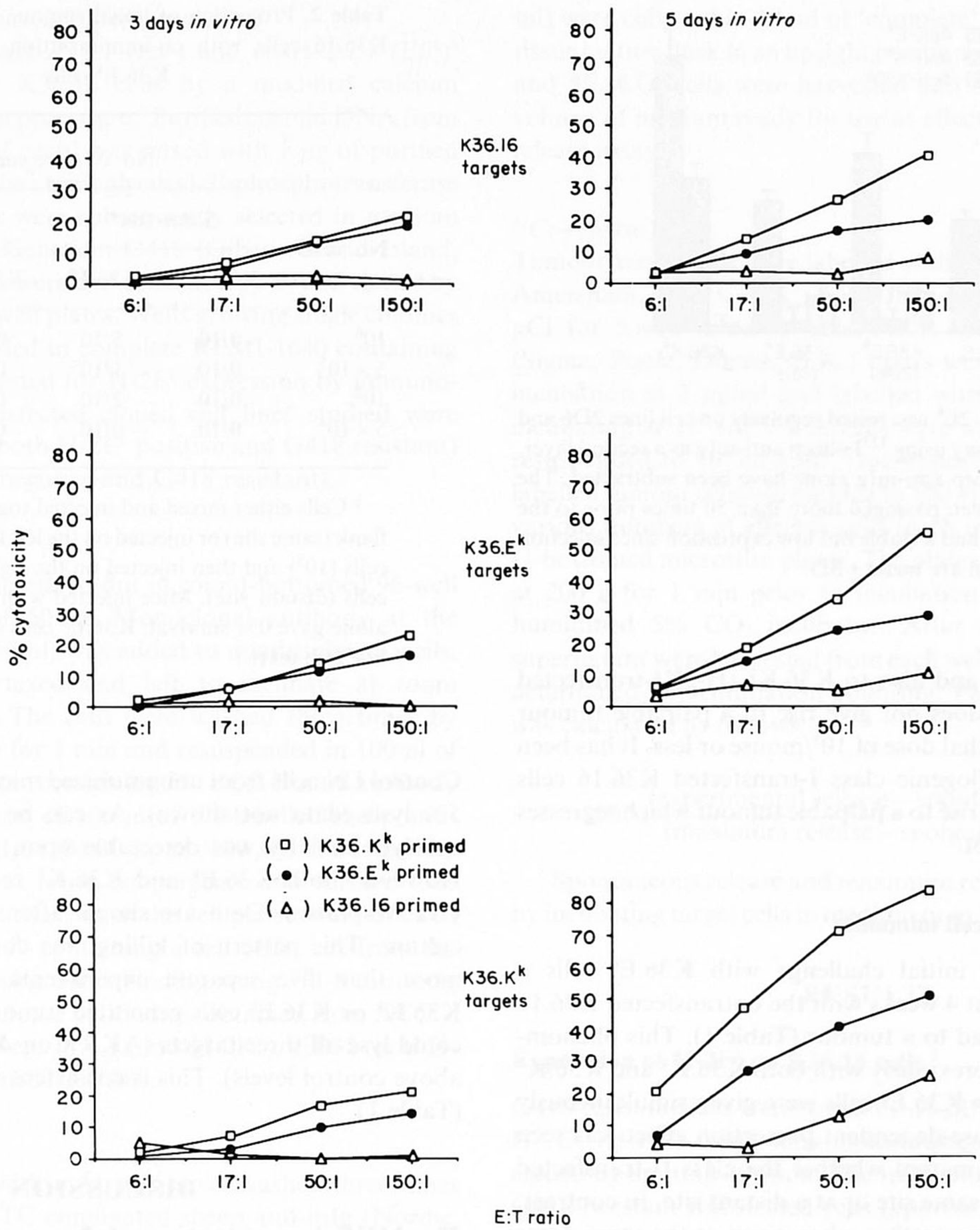

Figure 2. *In vitro* lysis of ^{51}Cr-labelled K36.16, K36.E^k (2D6) and K36.K^k target cells by lymph node cells from mice primed with tumour cells. AKR mice were immunized subcutaneously in the base of the tail with 10^6 viable tumour cells. Draining LN cells were removed 4 days later and cultured for 3 or 5 days before assay for CTL in ^{51}Cr-release assay.

antigen to responding T-helper cells. These, in turn, would act upon and enhance the recruitment of class I-restricted cytotoxic effector cells. It has been postulated that aberrant class II expression may result in the initiation of some autoimmune responses.[21]

One of the most puzzling, and as yet unresolved, aspects of tumour cell MHC transfection experiments is the apparent ability of mice which have been immunized with MHC class I-expressing cells derived from a class I loss mutant parent cell line to be resistant to subseuent challenge with the parent cell tumour.[3,8,22,23] It must be stressed that this is not a universal phenomenon.[11] Clearly if the induction of an immune response depended solely on the expression of an appropriate restriction element at the target cell level then the secondary immunity observed in such cases would not exist. Using transfection of both allogeneic and syngeneic class I genes it has been difficult to demonstrate *in vitro* syngeneic class I-restricted CTL to be present in the lymphoid tissue of apparently immune mice[3] unless protocols which reduce the level of suppressor cells are used[24] as here. Tumour-specific antibodies and natural killer cells have also not been found[12] and thus the mechanism of tumour cell destruction has not really been defined in these class I transfection experiments.

Using the well-studied K36.16 cell system we were able to derive clones of cells which had stable but low level expression of H-2E^k antigen (Fig. 1). The efficiency of successful transfection was low, probably because of the need for individual cells to incorporate Eα^k, Eβ^k and pTCF gene segments.

The tumourigenicity studies with A11 and 2D6 in many ways paralleled our previous work with H-2K^k-expressing K36.16 cells (K36.K^k).[3] That is, the majority of mice survived tumour challenge (Table 1). Such mice were also immune to a further challenge with a highly tumourigenic dose (5×10^5) of K36.16 cells, as was the case with K36.K^k immunized mice.[3] However, distinct differences between the K36.K^k and K36.E^k systems were noted. Firstly the dose of cells which were

tumourigenic was vastly different between the two groups. In the case of K36.K^k 10^5 cells gave 100% survival but higher doses led to significant mortality with 100% death at doses greater than 10^6/mouse. K36.E^k, on the other hand, gave 100% survival with doses as high as 4×10^6 cells/mouse. Secondly the appearance and progression of tumours was also different. K36.K^k challenge gave rise to no obvious tumour at sub-lethal doses (i.e. $\leqslant 10^5$/mouse) while mice dosed with the same number of K36.E^k cells gave obvious palpable tumours present from about 10 days post-challenge. These tumours progressed in size for a number of days until about Day 20 post-challenge, when they would begin to resolve, often disappearing within 2–3 days. Thirdly, and most importantly, the immune protection provided by K36.E^k challenge was more immediate than K36.K^k challenge. Simultaneous challenge with K36.K^k cells alongside K36.16 afforded no protection, whereas mice injected with K36.E^k cells at the same time as a highly tumourigenic dose of K36.16 cells often survived (Table 2). This phenomenon was dose dependent such that a high dose (10^6) of K36.E^k cells gave 50% protection with lower doses giving progressively less protection. Interestingly, the site of injection did not influence the result, which suggests that this is not simply a local phenomenon but must act systemically. These protection experiments parallel previous work with K36.16 cells transfected with the allogeneic class I antigen, H-2K^b (K36.K^b). Most K36.K^b clones could also provide some protection against a K36.16 challenge given 2 days earlier[3] even though only H-2K^b-specific CTL were detected *in vitro*. We have clearly shown here that CTL production does occur after K36.E^k challenge (Fig. 2). This significantly increases from Day 3 to Day 5 after *in vitro* culture and is not restricted to K36.E^k cells as K36.16 and K36.K^k targets are also lysed, as would be expected from *in vivo* data (Tables 1 and 2). Priming with K36.K^k cells also gave rise to good CTL response. However, this was not H-2K^k restricted, which is also consistent with the *in vivo* results. As expected K36.16 challenge gave rise to a minimal CTL response.

The exact nature of the mechanism of induction of tumour immunity by class I and class II gene transfection still remains to be resolved. Certainly, the early induction of $CD4^+$ T-helper cells has been shown to be important in other experimental models where tumour cells are lysed by $CD8^+$ T cells.[25] It has been postulated that class II-transfected tumour cells could directly present TSTA to $CD4^+$ helper cells.[17] The kinetics of the anti-tumour response may be very important. Thus the ability of K36.E^k cells to directly present tumour antigen to the immune system may allow the induction of non-H-2K^k-restricted CTL responses which are normally too slow to deal with a rapidly dividing tumour. The simplistic view that the transfection of the normally non-expressed H-2K^k molecule provides the missing restriction element for CTL responses against an unknown tumour antigen at the target cell level cannot really hold and needs further investigation. However, the strong and protective immune response generated by class II-transfected tumour cells as reported here may suggest a new method of tumour therapy.

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