Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 1762–1766, March 1990
Immunology

Transgenic mice expressing a soluble foreign H-2 class I antigen are tolerant to allogeneic fragments presented by self class I but not to the whole membrane-bound alloantigen

(major histocompatibility complex/protein secretion)

BERND ARNOLD*, MARTIN MESSERLE†, LIANE JATSCH*, GÜNTER KÜBLBECK*, AND ULRICH KOSZINOWSKI†

*Institute for Immunology and Genetics, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg, Federal Republic of Germany; and †Institute for Microbiology, Department of Virology, Oberer Eselsberg, 7900 Ulm, Federal Republic of Germany

Communicated by J. F. A. P. Miller, December 7, 1989

ABSTRACT **The properties of transmembrane and soluble transplantation antigens were compared with respect to the induction of tolerance and the selection of the T-cell repertoire. For this purpose, transgenic (*H-2^b* × *H-2^d*)F$_1$ mice were constructed that carry integrated copies of a modified *H-2K^k* gene resulting in the secretion from various cell types including thymocytes of soluble H-2K^k molecules. Despite the presence of H-2K^k antigen, these mice were still able to generate an H-2K^k-specific T-cell response. This response was comparable to that produced by normal littermates when stimulated with cells expressing membrane H-2K^k in a mixed lymphocyte reaction. In contrast, only transgenic mice failed to generate a cytolytic T-cell response to soluble H-2K^k antigen expressed by recombinant vaccinia virus and presented by the H-2D^b molecule. These data imply the presence of two populations of alloreactive cytolytic T cells. A small fraction of T cells recognizes alloantigen as antigenic peptide(s) presented by other major histocompatibility complex class I molecules and tolerance can be induced in this population by soluble alloantigen. The majority of T cells, however, require the whole cell membrane-expressed class I molecule for recognition. This population is not affected by tolerance induction to the soluble major histocompatibility complex class I molecule.**

In contrast to antibodies that can bind to soluble antigens such as free virus, receptors of T lymphocytes only recognize foreign antigens that are associated with a major histocompatibility complex (MHC) molecule on a cell membrane (1). This "MHC restricted antigen recognition" has become more understandable since the elucidation of the structure of a human MHC class I antigen, which has revealed a putative binding groove for antigen fragments (2). It is generally accepted that protein antigen (for instance, viral proteins) are processed by infected cells and presented to cytotoxic T lymphocytes (CTLs) by class I molecules (3). In the case of alloreactive responses, such as transplant rejections, it is still unclear whether processing and presentation of class I peptides by other class I molecules is a prerequisite for recognition by CTLs (4–7). If the number of alloreactive CTLs responding to a processed form of class I were a substantial fraction of the total number of CTLs reacting with class I antigen, this would have major implications for the induction of tolerance to class I antigens and on the modulations of transplant rejections. To study this question in more detail, we have recently designed a transgenic mouse model to test the influence of membrane-bound versus soluble alloantigen on the induction of tolerance and selection of the T-cell repertoire (8).

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Transgenic (*H-2^b* × *H-2^d*)F$_1$ mice were established carrying integrated copies of a modified *H-2K^k* gene, which led to secretion of soluble H-2K^k antigen by various cell types including thymocytes. Soluble H-2K^k was present in the sera of the transgenic animals (200 ng/ml). In spite of this, the transgenic mice were able to generate H-2K^k-specific CTL responses when stimulated with cell membrane-bound H-2K^k antigens (8). We have now analyzed the CTL responses of these animals to soluble H-2K^k by using either cells or recombinant vaccinia virus expressing soluble H-2K^k for immunization. We show here that normal mice immunized in such a way can mount a CTL response specific for an H-2K^k fragment and restricted by H-2D^b. However, transgenic mice expressing soluble H-2K^k are tolerant to MHC class I presented H-2K^k peptides.

MATERIALS AND METHODS

Construction of the Modified *H-2K^k* cDNA. A complete H-2K^k cDNA was obtained by recombination of two mutant H-2K^k cDNA clones (9): a 920-base-pair (bp) *Ava* II/*Bam*HI fragment of pcDk-1 contained the cDNA part 3′ to the *Ava* II site and a 300-bp *Ava* II/*Bam*HI fragment of pcDk-6 contained the cDNA part 5′ to the *Ava* II site. These two fragments were cloned into the *Bam*HI site of pUC19. The H-2K^d cDNA, in contrast to H-2K^k, contains a *Hin*dIII site exactly 3′ of the *a*3 domain coding region appropriate for the insertion of a stop codon. Hence, the *Pst* I/*Bam*HI 3′ fragment of the H-2K^k cDNA was replaced by the same fragment from an H-2K^k cDNA (obtained from P. Kourilsky, Paris). Insertion of a 22-bp stop codon into the *Hin*dIII site led to the modified H-2K^k cDNA C70. This was subsequently cloned into an expression vector containing the cytomegalovirus promoter and simian virus 40 poly(A) site (10).

Construction of Recombinant Vaccinia Virus. The modified H-2K^k cDNA C70 was cloned into the *Bam*HI site of the vaccinia virus recombinant vector CS43. To improve yield of plasmid DNA a 2.0-kbp *Hin*dIII/*Xho* I fragment of pGS62 (11) containing the vaccinia virus thymidine kinase gene driven by the 7.5-kbp promoter was inserted by blunt-end ligation into vector pUC9 cut with *Eco*RI and *Hin*dIII resulting in a plasmid, pCS43. Construction of the recombinant vaccinia virus followed established procedures (12) using the vaccinia virus strain Copenhagen and its temperature-sensitive mutant ts7 (13). Recombinant vaccinia virus containing the modified H-2K^k cDNA was termed VacK^{k}TM$^-$.

Cell Culture and Transfection of Cells. The fibroblast cell lines IT22-6 (H-2^q) and 3T3 (H-2^d) were used for DNA transfections. Cell culture conditions, DNA transfection, and

Abbreviations: CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; IL-2, interleukin 2.

analysis of transfectants by immunoprecipitation with monoclonal antibodies and gel electrophoresis were performed as described (14).

Generation of CTLs. Transgenic mice or nontransgenic littermates were immunized either with 5×10^7 transgenic spleen cells subcutaneously or with 5×10^7 plaque-forming units of recombinant vaccinia virus or wild-type virus Copenhagen intravenously. After 3–6 weeks, immune spleen cells (5×10^6 cells per ml in 24-well culture plates) were restimulated for 5 days *in vitro* with spleen cells of transgenic mice (2.5×10^6 cells per ml). The CTL population was expanded for another 3 days with 25 units per ml of recombinant interleukin 2 (IL-2). Alloreactive CTLs were generated in mixed lymphocyte cultures as described (14).

Target Cells and Cytolytic Assays. IC9 fibrosarcoma cells (15) were infected with 1 plaque-forming unit per cell of vaccinia virus strain Copenhagen. After 2 hr, the inoculum was removed, fresh medium was added, and cells were incubated overnight (12–18 hr). Target cells were treated with trypsin, labeled for 90 min with Na_2CrO_4, and used in a 4-hr standard chromium release assay (14).

RESULTS

Construction and Expression of the Modified H-2K^k cDNA. To test the putative antigenic difference between transmembrane and soluble class I protein, the appropriate cell transfectants and a recombinant viral vector were prepared. The modified H-2K^k cDNA C70, which resulted in a secreted H-2K^k antigen, was constructed in two consecutive steps (Fig. 1*A*). First, a *Pst* I/*Bam*HI fragment of the H-2K^k cDNA was replaced by the same fragment of an H-2K^d cDNA to introduce the *Hin*dIII restriction enzyme site exactly 3′ of the region coding for the *a*3 domain. In the second step, this *Hin*dIII site was used to insert a stop codon. The resulting modified cDNA encodes the signal sequence and the three outer domains of the H-2K^k antigen. There are four amino acid differences at the carboxyl-terminal end of the *a*3 domain in comparison to the wild-type H-2K^k introduced by the H-2K^d cDNA. The construct, called C70, was then cloned either into the expression vector pL31 containing the cytomegalovirus promoter and the simian virus 40 poly(A) site in pUC19 (10) (pC70), or into the vaccinia virus recombination vector CS43. The recombinant plasmid CS43–C70 was used together with Copenhagen strain DNA to transfect CV1 cells infected with the ts7 mutant strain of Copenhagen. The recombinant vaccinia virus VacK^{k}TM$^-$ containing the modified H-2K^k cDNA was selected after a temperature shift from 33°C to 39.5°C in the presence of BrdUrd (13).

The modified H-2K^k cDNA containing the stop codon 3′ of the region coding for the *a*3 domain (pC70) was transfected into IT22-6 cells. The resulting transfectants were screened for expression of mRNA in a dot blot using an H-2K^k-specific oligonucleotide (16). Positive cell clones were characterized by immunoprecipitation with monoclonal anti-H-2K^k antibodies and subsequent analysis by NaDodSO$_4$/PAGE. The antibody H100-27.55 (17) precipitated the C70 antigen from the cell lysate as well as from the cell supernatant (Fig. 1*B*, lanes 1 and 2). The antigen was found to be associated with β_2-microglobulin. The C70 antigen could also be precipitated with three other monoclonal antibodies, H-142-23, H100-5 (17), and R1-9.6 (18) that have been shown to react with different epitopes on the H-2K^k molecule. Therefore, we conclude that the exchange of the four amino acids in the carboxyl-terminal end of the *a*3 domain did not lead to a detectable alteration in the H-2K^k antigen. However, the introduction of a stop codon into the *Hin*dIII site led to an H-2K^k antigen that lacked the transmembrane and cytoplasmic regions, resulting in secretion.

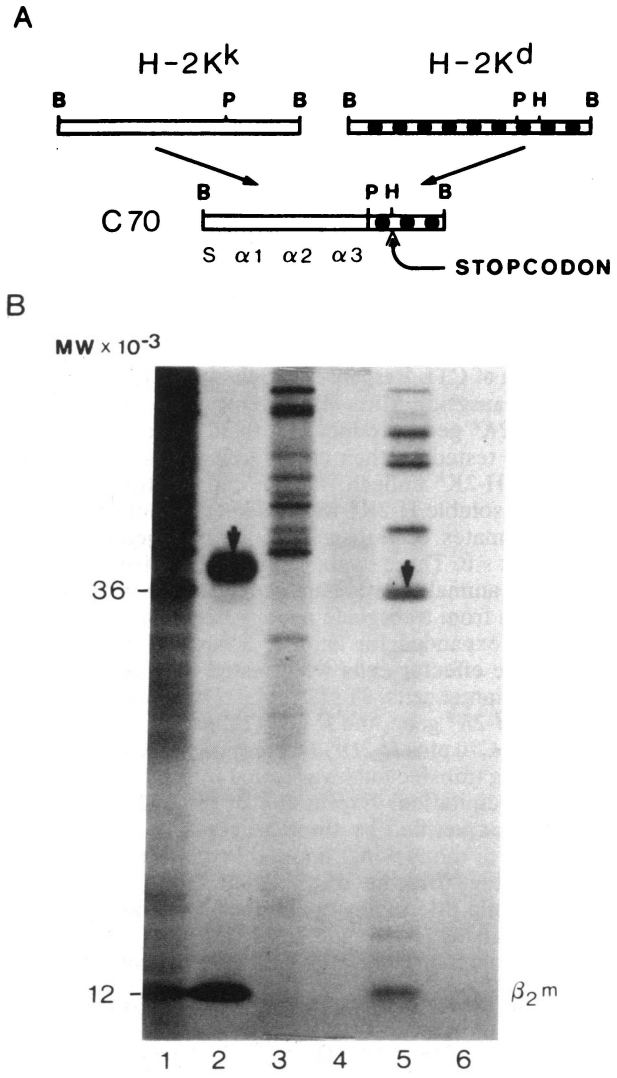

FIG. 1. Construction and expression of the modified H-2K^k cDNA C70. (*A*) The 340-bp *Pst* I (P)/*Bam*HI (B) fragment of the H-2K^k cDNA was replaced by the homologous fragment of an H-2K^d cDNA. The *Hin*dIII (H) site, which is exactly 3′ of the *a*3 domain coding region, was used to insert a stop codon. (*B*) The modified H-2K^k cDNA C70 was cloned in the expression vector pL31 containing the cytomegalovirus promoter region and the simian virus 40 poly(A) site. The resulting plasmid pC70 was transfected into IT22-6 fibroblasts. Transfectants were labeled with [^{35}S]methionine for 2 hr and were solubilized. Antigens in the cell lysate (lane 1) and the cell supernatant (lane 2) were precipitated with the monoclonal anti-H-2K^k antibody H100-27.55 and analyzed by NaDodSO$_4$/PAGE. The C70 DNA was also cloned in a recombinant vaccinia virus vector and IT22-6 cells were infected with either the wild-type or the recombinant vaccinia virus for 36 hr. Labeling of cells and immunoprecipitation were done as described above. Lanes 3 and 4, lysate and supernatant material from wild-type infected cells; lanes 5 and 6, material from cells infected with VacK^{k}TM$^-$. In lanes 1 and 5, the C70 antigen migrates according to an apparent molecular weight (MW) of 36,000 in association with β_2-microglobulin (β_2m). In the cell supernatant of the transfectants (lane 2), secreted C70 antigen exhibits an apparent molecular weight of 39,000.

IT22-6 cells were infected with 10 plaque-forming units per cell of either wild-type vaccinia virus strain Copenhagen or with recombinant virus VacK^{k}TM$^-$ for 1 hr. After an infection period of 36 hr, cells were labeled for 2 hr with [^{35}S]methionine and were then solubilized. As shown in Fig. 1*B*, the C70 antigen, associated with β_2-microglobulin, could be seen in lysates of cells infected with recombinant virus

$VacK^kTm^-$ after immunoprecipitation with the antibody H100-27.55 (Fig. 1*B*, lane 5). Apparently, the C70 antigen was not secreted in sufficient amounts for detection in cell supernatant (lane 6). As expected, the C70 antigen was not detected in lysates and supernatants of cells infected with wild-type virus (lanes 3 and 4).

Thus, infection of cells with the recombinant vaccinia virus leads to an H-2K^k antigen of the same size as the antigen obtained by transfection with the pC70 plasmid. The H-2K^k antigen could not be seen on the cell surface of either transfected or infected cells by fluorescence-activated cell sorter analysis with the antibody H100-27.55 labeled with fluorescein isothiocyanate.

Induction of CTL Responses to Soluble H-2K^k Using Spleen Cells of Transgenic Mice. Transgenic mice expressing the soluble *H-2K^k* gene product and their nontransgenic littermates were tested for their ability to mount a CTL response to soluble H-2K^k antigen. Spleen cells of transgenic mice expressing soluble H-2K^k were used to immunize nontransgenic littermates or transgenic mice by injecting 5×10^7 spleen cells s.c. Three to six weeks later, spleen cells of the immunized animals were restimulated *in vitro* with irradiated spleen cells from transgenic mice. After 4 days, activated T cells were expanded for another 3 days with recombinant IL-2. These effector cells were tested on a panel of transfectants as target cells. 3T3 fibroblasts were transfected with either the *H-2K^b* gene, the *H-2D^b* (19) gene, pC70, pC70 plus *H-2K^b*, or pC70 plus *H-2D^b*. Expression of the C70 antigen in the resulting transfectants was tested by mRNA analysis and immunoprecipitation. Expression of the H-2K^b and H-2D^b antigens was verified by fluorescence-activated cell sorter analysis with monoclonal antibodies (data not shown). As shown in Fig. 2*A*, the restimulated CTLs obtained from nontransgenic littermates could only lyse transfectants expressing both the H-2D^b and the C70 antigens, whereas CTLs obtained from transgenic mice could not lyse any of the target cells (Fig. 2*B*). Such effector cells, which apparently react with soluble H-2K^k restricted by the H-2D^b molecule, were not detected in mixed lymphocyte reactions (MLRs) from nontransgenic mice stimulated in a primary response by either spleen cells of transgenic mice expressing the soluble H-2K^k (Fig. 2*C*) or by spleen cells of B10.BR (H-2K^k) mice (Fig. 2*D*) expressing membrane-bound H-2K^k. Target cells expressing the intact membrane-associated H-2K^k antigen without the H-2D^b antigen were lysed by CTLs generated from both types of mice by stimulation with B10.BR spleen cells *in vitro* (Fig. 2 *D* and *E*).

It has been shown that class I antigens can be processed by cells and that peptides of this antigen can be presented by other class I molecules (4). Therefore, the IC9 fibrosarcoma cell line, which expresses only H-2D^b as a class I antigen (15), was transfected with the complete *H-2K^k* gene (15) to determine whether such cells could create the target structure for the CTL population reacting with the soluble H-2K^k in an H-2D^b restricted fashion. This CTL population, which was obtained from nontransgenic littermates as described above, could indeed react with IC9 cells expressing the transfected H-2K^k antigen, but it could not lyse untransfected IC9 cells or IC9 expressing the H-2K^d antigen (15) (Table 1). This result enabled us to localize the H-2K^k fragment that is associated with the H-2D^b antigen. IC9 transfectants were used that expressed H-2K^k/H-2K^d hybrid antigens (15). Target cells expressing only the α1 domain of H-2K^k within the H-2K^d molecule could be lysed by the CTL population, whereas target cells expressing α2 and α3 of H-2K^k within the H-2^d molecule were not lysed. All the IC9 cell lines used were equally susceptible to lysis by anti-H-2D^b-specific CTLs (data not shown).

Induction of CTL Responses to Soluble H-2K^k Using the Recombinant Vaccinia Virus. To confirm the results described above, transgenic and nontransgenic mice were immunized with the recombinant vaccinia virus expressing

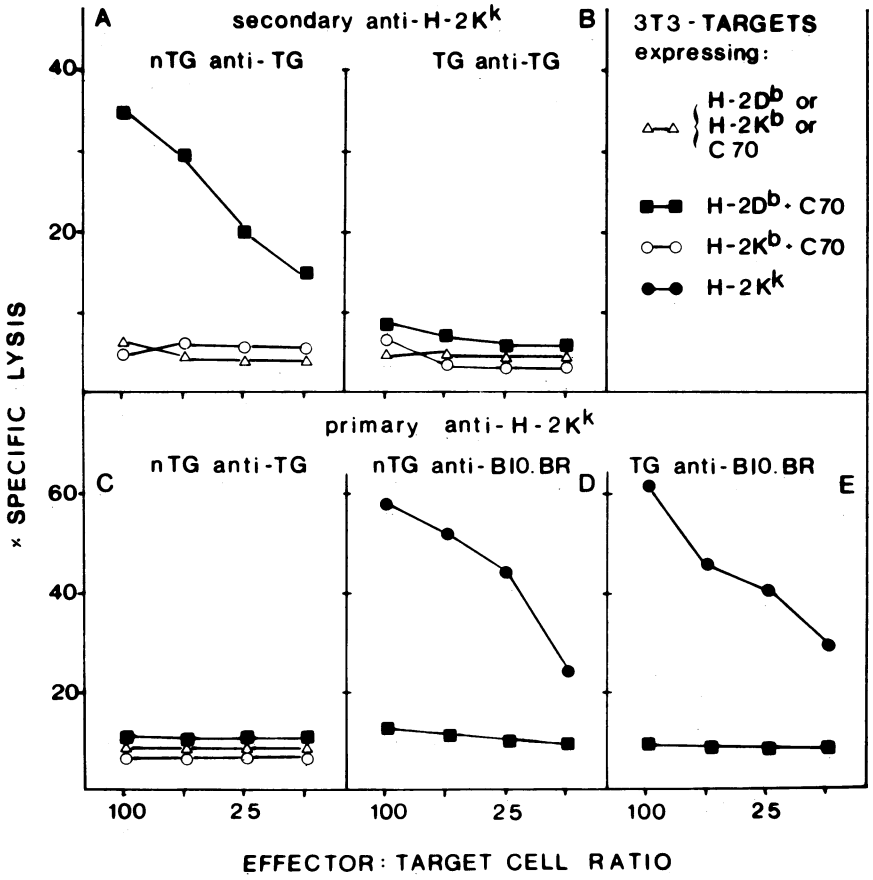

FIG. 2. CTL responses of C57BL/6 transgenic mice (TG) and nontransgenic littermates (nTG) against H-2K^k antigens. Mice were immunized with 5×10^7 spleen cells s.c. After 3–6 weeks, spleen cells of the immunized animals were restimulated with irradiated spleen cells of transgenic mice for 4 days and activated T cells were expanded for another 3 days with recombinant IL-2. Effector populations from nontransgenic (*A*) and transgenic (*B*) mice were tested on a panel of 3T3 transfectants as indicated. CTLs obtained from primary MLRs were also tested: nontransgenic littermates anti-transgenic mice (*C*), or anti-B10.BR (H-2^k) (*D*) mice, and transgenic mice anti-B10.BR (*E*). Curves indicate results of the analysis of one representative mouse. Ten mice (*A* and *B*) or four mice (*C–E*) were analyzed.

Table 1. Localization of the H-2K^k fragment recognized by CTLs in association with H-2D^b

Target cells	Allele of K^d/K^k hybrid antigen domains			% specific lysis	
	α1	α2	α3	E/T = 100:1	E/T = 50:1
IC^9	—	—	—	6	5
9K^k-34	*k*	*k*	*k*	31	18
9K^d-2	*d*	*d*	*d*	4	4
9C31-3	*d*	*k*	*k*	5	2
9C25-4	*k*	*d*	*d*	35	23

Values indicate results of the analysis of one representative mouse. Eight mice were tested individually. The percentages of lysis are mean values of triplicate wells analyzed. E/T, effector/target cell ratio. The IC9 fibrosarcoma line expresses only H-2D^b as a class I antigen. CTLs were generated as described in the legend to Fig. 2.

soluble H-2K^k. MHC class I molecules expressed by vaccinia virus vectors have been successfully applied for the generation of CTL responses to alloantigen (20). Irradiated spleen cells of transgenic animals were used for restimulation *in vitro* 3–6 weeks later. CTLs were expanded with recombinant IL-2 as mentioned above. CTL populations isolated from nontransgenic littermates could lyse IC9 cells expressing the H-2K^k antigen (Fig. 3*A*). CTL populations obtained from transgenic mice did not show this activity (Fig. 3*B*). The same results were obtained with the above-mentioned 3T3 transfectants as targets (data not shown). On the other hand, if spleen cells from mice immunized with recombinant vaccinia virus VacK^{k}TM$^-$ were tested 6–8 days after priming for their ability to lyse wild-type vaccinia virus-infected target cells, no difference could be observed in the degree of specific lysis by transgenic and nontransgenic animals (Fig. 3 *C* and *D*). We can, therefore, conclude that nontransgenic littermates can respond to recombinant vaccinia virus in activating CTLs specific for an H-2K^k fragment in association with H-2D^b. Transgenic mice, however, are tolerant to this target structure. Evidence for effective immunization of both types of mice was demonstrated by the primary *in vivo* anti-vaccinia virus response.

DISCUSSION

We have recently reported that transgenic mice expressing a foreign class I antigen in soluble form could still react with the same high frequency of CTL precursors to the membrane-bound alloantigen as normal mice (8). We could, however, not exclude that the lack of tolerance toward the membrane antigen was based on the fact that the concentration of the antigenic protein in the serum (200 ng/ml) might have been insufficient to induce any tolerance. We have therefore extended the analysis of these transgenic mice to study their reactivity to the soluble form of the class I antigen. H-2K^k-specific, H-2D^b-restricted CTLs were induced in nontransgenic littermates by immunization with either spleen cells of transgenic mice or by recombinant vaccinia virus expressing a soluble form of the H-2K^k antigen. A peptide fragment of the *a*1 domain of H-2K^k is recognized in association with H-2D^b but not with H-2K^b under these conditions. The observed specific cytolytic activity is smaller than the one resulting from a primary anti-H-2K^k MLR and requires both *in vivo* priming and restimulation *in vitro*.

Our results confirm that normal mice can respond in two ways to MHC class I antigens: CTLs can be generated

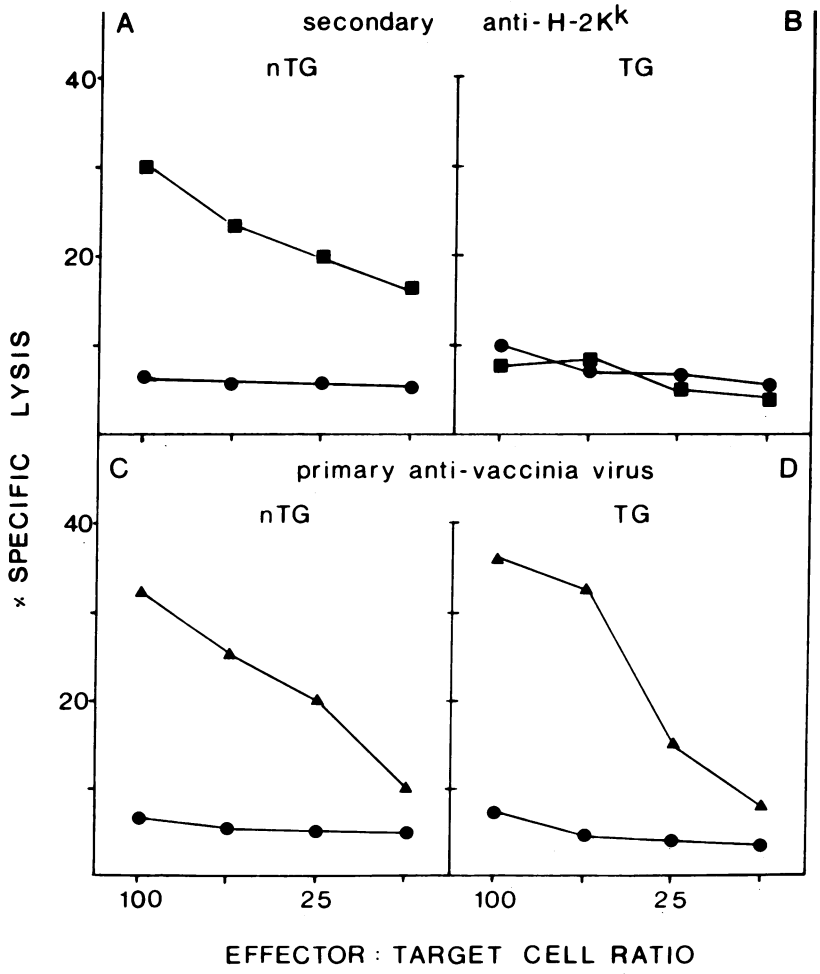

FIG. 3. Generation of CTLs by immunization with recombinant vaccinia virus VacK^{k}TM$^-$. Mice were immunized with 5×10^7 plaque-forming units of virus VacK^{k}TM$^-$ i.v. and were killed either after 6–8 days for a primary anti-vaccinia virus response or after 3–6 weeks for the analysis of an anti-H-2K^k response. In the second case, spleen cells were restimulated with irradiated spleen cells from transgenic mice for 4 days. CTLs were expanded for another 3 days with recombinant IL-2. CTLs of nontransgenic (nTG) (*A*) or transgenic (TG) (*B*) mice were tested on IC9 (●) and 9K^k-34 (■) target cells. For the primary anti-vaccinia virus response, spleen cells of nTG (*C*) and TG (*D*) were tested on either IC9 (●) or on infected IC9 target cells. Targets were infected with 10 plaque-forming units of vaccinia virus per cell for 1 hr and after removal of the inoculum were incubated overnight (▲). Curves indicate the result of the analysis of one representative mouse. Twelve mice of each type (*A* and *B*) or five mice of each type (*C* and *D*) were tested individually.

reacting either with an intact class I antigen (H-2K^k) most likely associated with self peptides (21) or to a peptide fragment of this class I antigen (H-2K^k) in association with a membrane-bound class I antigen, such as H-2D^b (4–7). The mice transgenic for the soluble H-2K^k are still reactive toward the membrane-bound H-2K^k but they are clearly tolerant for H-2K^k peptide(s).

These findings have three major implications. First, soluble class I antigens cannot induce tolerance by direct binding to the receptors of T cells reactive with the intact form of the membrane-associated antigen. It has been shown that soluble class I antigen could interfere with the stimulation of an alloreactive T-cell hybridoma *in vitro* (22). This T-cell hybridoma was reactive to the membrane-bound H-2K^b antigen independent of self MHC proteins. However, soluble H-2K^b molecules could only inhibit stimulation of the T-cell hybridoma under suboptimal conditions using H-2K^{bm} mutant antigens. Therefore, these data do not contradict our *in vivo* results.

Second, MHC molecules can appear in two distinct antigenic structures. They can either present peptides themselves or they can be processed and presented in peptide form by self MHC molecules. CTLs generated in a primary MLR can lyse transfectants expressing the relevant class I antigen irrespective of the haplotype of the transfected cell line (14, 19). Thus, the intact class I antigen is essential for recognition. On the other hand, CTLs that react with class I peptides associated with self class I molecules have been described in several systems (4–7). However, the percentage of these T cells must be low within the alloreactive CTL precursor population, because no CTLs could be found specific for soluble H-2K^k in association with H-2D^b in primary anti-B10.BR bulk cultures. Only by immunization with transfectants expressing soluble H-2K^k or with recombinant vaccinia virus could this H-2K^k-specific H-2D^b-restricted response be obtained. The transgenic mice are tolerant for this type of T-cell specificity but can nevertheless respond with a high CTL precursor frequency in a primary anti-H-2K^k MLR that is indistinguishable from normal mice (8).

Third, an additional aspect of this work is related to the finding that tissue-specific, nonthymic expressed class I antigens in transgenic mice can lead to tolerance to this class I protein (23, 24). In the two systems that have currently been described, usage of an insulin promoter resulted in expression of H-2K^b in the pancreas (23), while a metallothionein promoter caused expression in the liver (24). However, it has been suggested that the observed tolerance could still be based on a thymic event. Class I antigens could be shedded from the respective tissue, taken up by cells migrating to the thymus, and presented by self class I and II molecules. Our results contradict this suggestion. Even if peptides of the class I antigens that are expressed extrathymically could be presented in the thymus, only the small fraction of the alloreactive CTL precursor pool that is specific for a class I peptide could be made tolerant. The majority of alloreactive CTL precursors reacting with the intact class I antigen should, however, be unaffected unless extrathymic tolerance mechanisms function.

This work was supported by the Deutsche Forschungsgemeinschaft (B.A., Ar 152/2-1; U.K., SFB 322,B6).

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