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Immunology

Alloreactive immune responses of transgenic mice expressing a foreign transplantation antigen in a soluble form

(major histocompatibility complex/class I genes/tolerance/protein secretion)

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ABSTRACT **Transfection of cells with the *H-2K*^{k} gene lacking the transmembrane and cytoplasmic segments resulted in secretion of the H-2K^k protein, as determined by immunoprecipitation with monoclonal anti-H-2K^k antibodies. Transgenic (*H-2*^{b} × *H-2*^{d})F$_1$ mice were established carrying integrated copies of the modified *H-2K*^{k} gene. Expression of the soluble H-2K^k antigen in the transgenic mice was demonstrated in cell supernatants of biosynthetically labeled splenic and thymic Con A blasts as well as bone marrow-derived macrophages. Soluble H-2K^k molecules were also present in the sera of the transgenic animals. No cell-surface expression of the H-2K^k antigen could be observed. In spite of the presence of the soluble H-2K^k molecules in the transgenic mice, the animals were able to generate H-2K^k-specific cytolytic T cells as well as antibody responses when stimulated with cell-surface-bound H-2K^k antigens. These responses were indistinguishable from those of the nontransgenic littermates. Possible explanations for the observed lack of tolerance are discussed.**

The selection of the T-cell repertoire results in T cells that recognize foreign antigens only in association with the major histocompatibility complex (MHC) antigens (1). These T cells react also with high frequency with allogeneic MHC antigens, possibly due to crossreactivity (2). It is assumed that this selection takes place in the thymus, where T cells with high affinity for self MHC antigens are eliminated, resulting in tolerance for self MHC determinants. The mechanism of these selection processes is not understood. It is known that the presence of MHC molecules in the thymus is mandatory, but it is not clear if these MHC molecules have to be expressed as integral membrane proteins or if soluble MHC antigens are sufficient for induction of tolerance and education of T cells. Transgenic mice expressing a soluble "foreign" transplantation antigen should help to clarify this question.

A prerequisite for this study is the conversion of membrane-associated proteins into secreted forms, a process that can be achieved by deletion of gene segments encoding the transmembrane portion of the protein (3). In this paper, we describe such a modification of the *H-2K*^{k} gene that leads to secretion of H-2K^k molecules by transfected cells. We also show that transgenic mice carrying the modified gene secrete the soluble H-2K^k protein into their serum. Immunological studies demonstrate that the presence of soluble H-2K^k proteins in these mice does not cause tolerance in cytolytic T lymphocyte (CTL) precursors and B cells specific for cell-surface-bound H-2K^k antigens.

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MATERIALS AND METHODS

Construction of the Modified *H-2K*^{k} Gene. The *H-2K*^{k} gene and its deletion subclone containing exons I–IV have been described (4, 5). The gene organization of β_2-microglobulin was published elsewhere (6). The genomic clone of β_2-microglobulin was a gift of M. Steinmetz (Hoffmann–La Roche, Basel). The 1400-base-pair (bp) *Eco*RI/*Xba* I fragment of the Moloney murine leukemia virus enhancer (7) was inserted into the plasmid pUC 19. The resulting plasmid was used to clone the following two DNA fragments simultaneously: (*i*) The H-2K^k subclone containing exons I–IV was treated with *Cla* I endonuclease, made blunt-ended with the Klenow polymerase, and partially digested with *Xba* I. This treatment led to a 3450-bp fragment. (*ii*) The β_2-microglobulin gene was cut with *Kpn* I, made blunt-ended, and treated with *Hin*dIII. In this way, a 3000-bp fragment was obtained containing exons III and IV. Cloning of these two fragments into the *Xba* I/*Hin*dIII sites of the enhancer-containing plasmid led to the modified *H-2K*^{k} gene C50.

Cell Culture and DNA Transfection of Cells. The fibroblast cell line 1T22-6 (*H-2*^{q}) was used for DNA transfections. Cell culture conditions, DNA transfection method, and analysis of transfectants by immunoprecipitation with monoclonal antibodies and gel electrophoresis have been described (8).

Construction of Transgenic Mice. Eggs were collected from (C57BL/6 × DBA/2)F$_1$ females mated with (C57BL/6 × DBA/2)F$_1$ males. Embryos were maintained *in vitro* in Whitten's medium (9). DNA (0.01 mg/ml) was injected into the male pronucleus of the developing embryos as described (9). Surviving embryos were transferred into oviducts of randomly bred Swiss females mated with vasectomized Swiss males the night before (9). Mice were tested for the integration of the transgene by Southern blot analysis of the placenta and tail DNA (9).

ELISA for Detection of Soluble H-2K^k Antigen. The ELISA was performed as described (10). The following four steps were used: (*i*) overnight incubation of polyvinylchloride microtiter plates with the mouse monoclonal anti-H-2K^k antibody H142-23 (11); (*ii*) addition of the test samples; (*iii*) incubation with the rat monoclonal anti-H-2K^k antibody R1-9.6 (12); (*iv*) incubation with a peroxidase-labeled goat anti-rat immunoglobulin antiserum (Jackson ImmunoResearch, West Grove, PA). The substrate reaction with *o*-phenylenediamine was measured in a Titertek-Multiscan at 492 μm.

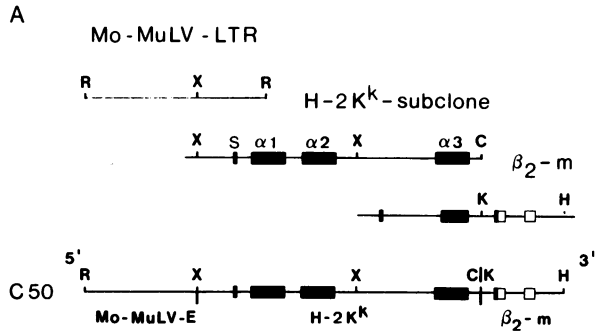
Mixed Lymphocyte Cultures. Alloreactive CTL were generated in mixed lymphocyte culture by stimulation of 10^7 spleen cells of transgenic mice or their nontransgenic littermates with 10^7 irradiated B10.BR spleen cells as described (8). The limiting dilution system to estimate the precursor

Abbreviations: CTL, cytolytic T lymphocyte(s); MHC, major histocompatibility complex.

frequency of alloreactive CTL has been described in detail (13).

RESULTS

Construction and Expression of the Modified *H-2K*^{k} Gene. The modified *H-2K*^{k} gene (C50), which leads to a secreted H-2K^k antigen, was constructed from three components (Fig. 1*A*). A deletion subclone of the *H-2K*^{k} gene, which contained the promotor region and the exons encoding the signal sequence and the three outer domains, was combined with exons III and IV of β_2-microglobulin encoding three amino acids, the termination codon, and the 3′ untranslated region. The Moloney murine leukemia virus enhancer was inserted 5′ into this gene construct.



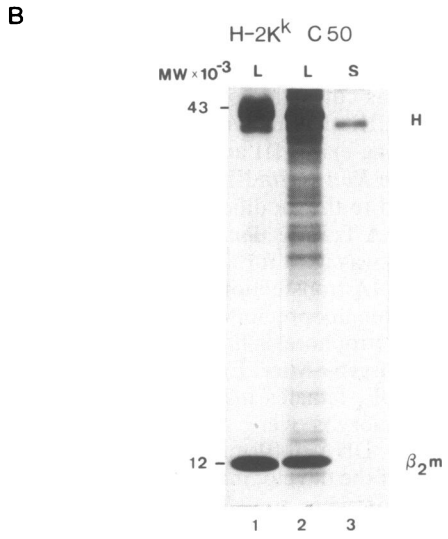


FIG. 1. Construction and expression of the modified *H-2K*^{k} gene C50. (*A*) The C50 plasmid was obtained by combining three components in pUC 19. First, the 1400-bp *Eco*RI(R)/*Xba* I (X) fragment containing the Moloney murine leukemia virus enhancer (Mo-MuLV-E) was isolated from the whole 5′ Moloney murine leukemia virus long terminal repeat (Mo-MuLV-LTR) and cloned in pUC 19. Second, the 3450-bp *Xba* I (X)/*Cla* I (C) fragment of an H-2K^k subclone and the 3000-bp *Kpn* I (K)/*Hin*dIII (H) fragment of β_2-microglobulin (β_2m) were inserted simultaneously between the *Xba* I and the *Hin*dIII sites of the Mo-MuLV-E-containing pUC 19 plasmid. The *Cla* I (C) and the *Kpn* I (K) sites had been blunt-ended. The C50 gene contains, besides the enhancer element, exons I–IV of the *H-2K*^{k} gene encoding the signal sequence and the three outer domains α1, α2, α3 and exons III and IV of the β_2-microglobulin gene encoding three amino acids, the stop codon, and the 3′ untranslated region. (*B*) 1T 22-6 fibroblasts were transfected with the *H-2K*^{k} or the C50 gene. Transfectants were labeled with [^{35}S]methionine for 2 hr and solubilized. Antigens in the cell lysate (L, lanes 1 and 2) or cell supernatant (S, lane 3) were precipitated with the monoclonal anti-H-2K^k antibody H100-27.55 and analyzed by NaDodSO$_4$/PAGE. The positions of the heavy chain (H) and the light chain (β_2m) of the H-2 antigens are indicated. Soluble C50 antigen could be identified in the cell supernatant (lane 3) with an apparent molecular weight (MW) of 40,000.

C50 was transfected into IT22-6 fibroblasts. The resulting transfectants were screened for expression of mRNA in a dot blot using an H-2K^k-specific oligonucleotide (14). The positive cell clones were characterized by immunoprecipitation with monoclonal anti-H-2K^k antibodies and subsequent analysis by NaDodSO$_4$/PAGE. For example, the antibody H100-27.55 (11) could precipitate the C50 antigen from the cell lysate as well as from the cell supernatant (Fig. 1*B*, lanes 2 and 3). The antigen is associated with β_2-microglobulin (lane 2). The very faint band of β_2-microglobulin in the cell lysate (lane 3) indicates a rapid exchange of the mouse β_2-microglobulin with the bovine β_2-microglobulin of the fetal calf serum (15), since the antibody H100-27.55 does not react with the isolated H-2K^k chain. The C50 antigen has a smaller apparent molecular weight than the membrane-bound H-2K^k antigen (lane 1). The difference, however, is not as big as expected according to the number of amino acids, which could be due to differential splicing at the 3′ end of the C50 gene. The C50 antigen could also be precipitated with three other monoclonal antibodies [H142-23, H100-5 (11), R1-9.6 (12)] that have been shown to react with different epitopes on the H-2K^k molecule.

Therefore, we conclude that the secreted H-2K^k antigen is associated with β_2-microglobulin and contains the conformational determinants seen by four different monoclonal antibodies.

Transgenic Mice Carrying the Modified *H2K*^{k} Gene. The 8000-bp *Eco*RI/*Hin*dIII fragment of C50 was used for microinjections. A founder mouse was shown by Southern blot analysis of tail DNA to carry the gene (Fig. 2). By analyzing the offspring of this female, it was shown that several copies of the C50 gene had been integrated into the X chromosome and were stably transmitted in a sex-linked inheritance.

Expression of Soluble *H-2K*^{k} in Transgenic Mice. Transgenic (C57BL/6 × DBA/2)F$_2$ mice were analyzed for expression of the integrated gene on the mRNA and protein levels. mRNA of different organs was isolated and analyzed in a dot blot assay as described (16). Fig. 3*A* shows such an analysis with H-2K^k (14) and H-2K^b-specific (17) oligonucleotides as probes. The amount of mRNA specific for soluble

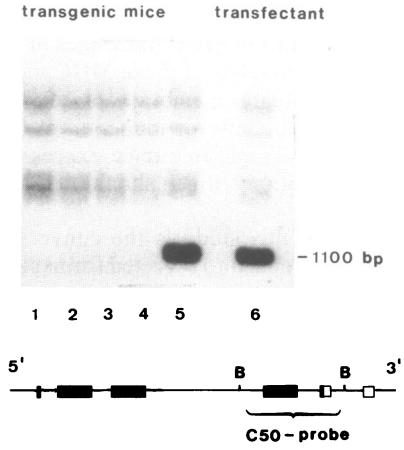


FIG. 2. Identification of (C57BL/6 × DBA/2)F$_2$ mice having integrated the C50 gene in their genome by Southern blot analysis. DNA was extracted from tail biopsy samples or from C50-transfected cells and digested with *Bam*HI. The 1100-bp *Bam*HI (B) fragment of the C50 gene was used as a probe. With this probe transgenic mice (lane 5) could be distinguished from their nontransgenic littermates (lanes 1–4), since the smallest *Bam*HI fragment of the endogenous class I genes crossreacting with the C50 probe was 2200 bp.

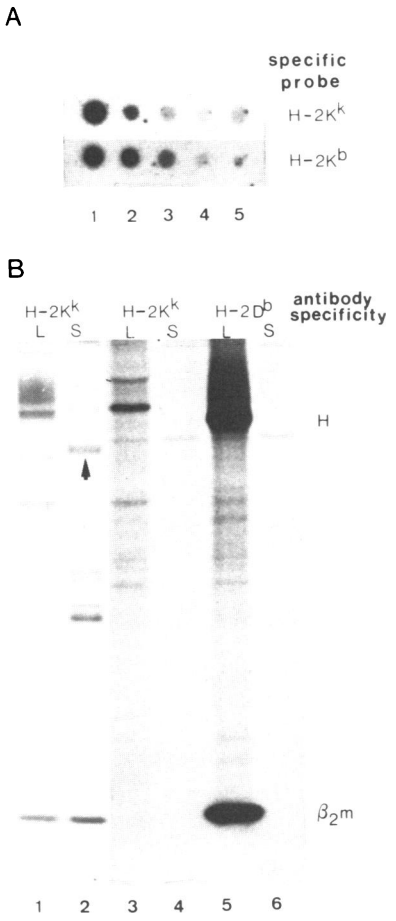


FIG. 3. Expression of the modified *H-2K^k* gene in transgenic mice. (*A*) RNA was isolated from liver (*1*), spleen (*2*), kidney (*3*), heart (*4*), and brain (*5*) of the transgenic mouse 1421. RNA (3 μg) was applied to nitrocellulose filters and hybridized with end-labeled H-2K^k- and H-2K^b-specific oligonucleotides. (*B*) Thymocytes of the transgenic mouse 1421 (lanes 1 and 2) or of the nontransgenic littermate 1424 (lanes 3–6) were stimulated with Con A (Miles, Rehovot, Israel) at 10 μg per 10^6 cells per ml. Lysates (L) and cell culture supernatants (S) of the Con A blasts were used for immunoprecipitation with the monoclonal antibodies 100–27.55 specific for H-2K^k (lanes 1–4) and B22-249 specific for H-2D^b (lanes 5 and 6) and NaDodSO$_4$/PAGE analysis. Thymocytes of the transgenic mouse 1421 could secrete soluble H-2K^k (arrow, lane 2) associated with β_2-microglobulin (β_2m). The *b* allele of β_2-microglobulin apparently does not exchange as rapidly with bovine β_2-microglobulin in the serum as the *a* allele (compare Fig. 1*B*, lane 3).

H-2K^k in liver (1), spleen (2), kidney (3), heart (4), and brain (5) was comparable to that of the mRNA for the intrinsic H-2K^b antigen.

Thymocytes of the same transgenic animal were stimulated with Con A and the resulting blast cells were used for immunoprecipitation with antibody H100-27.55 and NaDodSO$_4$/PAGE (Fig. 3*B*). The stimulated thymocytes could secrete H-2K^k (lane 2). In control studies, no H-2K^k could be found when thymocytes of nontransgenic littermates were analyzed in the same way (lane 4). The latter Con A blasts had been correctly stimulated as shown by the immunoprecipitation with the H-2D^b-specific antibody B22-249 (18) (lanes 5 and 6). Similar results were obtained with bone-marrow-derived macrophages (data not shown).

To verify the appearance of soluble H-2K^k in the serum of the transgenic mice, an ELISA technique was used. Two H-2K^k-specific monoclonal antibodies were used that do not block the binding of each other and therefore are directed against distinct epitopes (11). The capture antibody attached to the plate was mouse-derived (H141-23), the one to identify the bound antigen a rat derived (R1-9.6) antibody. Sera of the transgenic animals gave positive signals in the ELISA, in most cases 5- to 6-fold above control values (Table 1). These results could be confirmed by using ascites fluid of transgenic mice induced with Ehrlich ascites cells (data not shown). Purification of soluble H-2K^k protein from ascites fluid of transgenic animals led to the estimate of ≈200 ng of H-2K^k protein per ml of body fluid.

Thus, the data obtained with three different techniques demonstrate that various cell types of the transgenic mice were able to produce soluble H-2K^k and that this material is present in the serum of these mice. No cell-surface expression of the H-2K^k antigen could be observed by fluorescence-activated cell sorter analysis.

H-2K^k-Specific CTL Response of Transgenic Mice Expressing Soluble H-2K^k. Spleen cells of transgenic mice with soluble H-2K^k in the serum were stimulated with irradiated B10.BR spleen cells under mixed lymphocyte culture conditions. CTL generated in these primary bulk cultures were analyzed on parental IT 22-6 cells and on IT 22-6 cells transfected with either the *H-2K^k* or the *H-2D^k* gene. As shown in Fig. 4, CTL responses against H-2K^k and H-2D^k antigens were obtained that were indistinguishable from the responses of nontransgenic littermates. In addition, transgenic and nontransgenic animals did not show differences in their precursor frequencies for either H-2K^k- or H-2D^k-specific CTL (1/200–1/300) as revealed by limiting dilution analysis (13).

H-2K^k-Specific Antibody Response of Transgenic Mice Expressing Soluble H-2K^k. To determine whether mice expressing soluble H-2K^k could mount an antibody response to membrane-bound H-2K^k, 10 transgenic mice and 10 nontransgenic littermates were immunized three times with B10.A(4R) spleen cells. After the third immunization, sera of these mice were analyzed in a cellular radioimmunoassay using 1T22-6 cells transfected with the *H-2K^k* gene (Fig. 5). The values obtained with the immune sera were compared with the preimmune sera. A broad spectrum of reactivities could be seen. A few animals did not respond at all. Others showed 5- to 30-fold increases in the antibody-binding assay when compared to controls. Again, however, no difference was detectable between transgenic and nontransgenic animals.

Ontogeny of Expression of Soluble H-2K^k. To clarify whether the normal CTL and antibody responses against membrane-associated H-2K^k reported above could be explained by the late appearance of soluble-H-2K^k protein during ontogeny, offspring of transgenic males and normal females were tested. Day 18 or 19 embryos and the corresponding placentas were taken. Placental DNA was isolated. In addition, spleen and thymus of embryos were stimulated with Con A and tested for expression of soluble H-2K^k by

Table 1. Detection of soluble H-2K^k antigen in the serum of transgenic mice by ELISA

Mouse	Transgene* detected	ELISA extinction† serum 1:20
1409	–	0.19
1416	+	0.95
1419	+	0.33
1420	+	1.01
1421	+	1.11
1424	–	0.18
NMS		0.18

*By Southern blot analysis of tail DNA (see Fig. 2).
†Antibodies used were H142-23 for coupling on plates and R1-9.6 and goat anti-rat antiserum peroxidase labeled for identification of the soluble H-2K^k antigen.

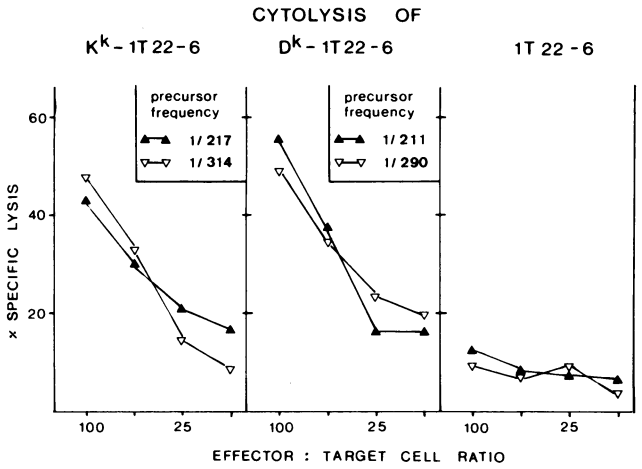


FIG. 4. CTL response of transgenic mice against H-2K^k antigens. Spleen cells of transgenic mice expressing soluble H-2K^k (▲) and of nontransgenic littermates (▽) were stimulated with irradiated spleen cells of B10.BR mice. On day 5, CTL were tested on ^{51}Cr-labeled 1T22-6 cells and 1T22-6 transfectants expressing the H-2K^k or H-2D^k antigen. The percentages of lysis are mean values of triplicate wells analyzed. Curves represent results of the analysis of one mouse. Ten mice of each type were tested individually. CTL precursor frequencies in individual mice were determined after limiting dilution analysis (data not shown) by the minimal κ^2 method (19). Experiments for which the two-tailed probability was >0.05 were accepted. The 95% confidence limits for the target K^k-1T22-6 were 1/164–1/321 (▲, transgenic) and 1/239–1/460 (▽, nontransgenic) and for the target D^k-1T22-6 the limits were 1/169–1/280 (▲) and 1/217–1/441 (▽).

immunoprecipitation. Whenever the transgene was detected in the placental DNA, expression of the soluble H-2K^k protein could also be found (Fig. 6). This finding is also in agreement with the analysis of mRNA isolated from the embryos showing positive signals in a dot blot with the H-2K^k-specific oligonucleotide (data not shown).

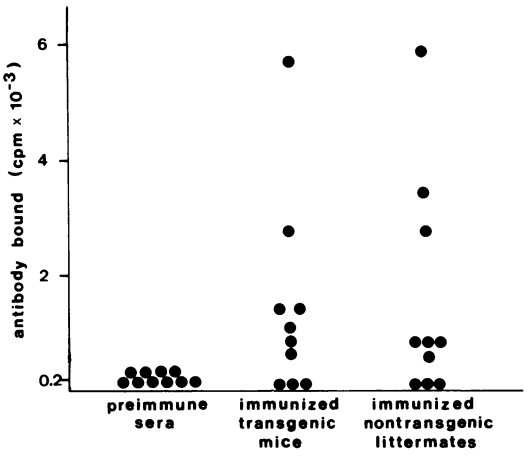


FIG. 5. Antibody response of transgenic mice against membrane-associated H-2K^k antigen. Transgenic mice and nontransgenic littermates were immunized i.p. once with 1×10^7 and at 3-week intervals again two times with 5×10^7 B10.A (4R) spleen cells. Preimmune sera and immune sera of both types of mice were tested in a cellular radioimmunoassay using H-2K^k-transfected 1T22-6 cells. Antibody binding was detected with ^{125}I-labeled protein A. The mean values of triplicate wells are given. One dot represents data of one antiserum. The transgenic mouse that produced the best anti-H-2K^k antiserum was used for the generation of spleen Con A blasts. Immunoprecipitation with the monoclonal anti-H-2K^k antibody H100-27.55 and NaDodSO$_4$/PAGE analysis of the cell supernatant confirmed that this mouse still produced soluble H-2K^k (data not shown).

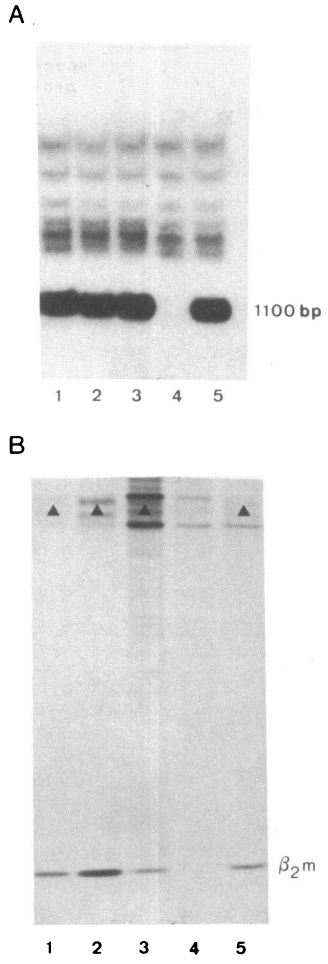


FIG. 6. Expression of soluble H-2K^k early in ontogeny. Day 18 or 19 embryos from matings of transgenic males and nontransgenic females and the corresponding placentas were isolated. (*A*) *Bam*HI-digested placenta DNA was analyzed by Southern blotting as described in Fig. 2. (*B*) Stimulation of spleen cells and thymocytes of the embryos, labeling of the blast cells with [^{35}S]methionine, immunoprecipitation of the cell lysates with the antibody H100-27.55, and NaDodSO$_4$/PAGE was performed as described in Fig. 1*B* and Fig. 3*B*. Whenever the transgene was detectable (lanes 1, 2, 3, and 5) soluble H-2K^k (faint bands) and β_2-microglobulin (β_2m) (strong bands) could be seen. The antibody H100-27.55 does not crossreact with isolated β_2-microglobulin. The exposure time of the autoradiograph was 4 weeks.

DISCUSSION

The present paper describes a system that was designed to test the influence of membrane bound versus soluble alloantigen in the induction of tolerance and selection of the T-cell repertoire.

We have substituted the 3′ end of the *H-2K^k* (exons V–VIII) by the 3′ end of the β_2-microglobulin gene (exons III and IV) to obtain a secreted transplantation antigen. The resulting H-2K^k antigen was associated with β_2-microglobulin and could be identified in cell supernatants with four monoclonal anti-H-2K^k antibodies directed against distinct epitopes. According to these criteria, the conformation of the secreted molecule is indistinguishable from that of the membrane-associated form. Other investigators have obtained a soluble class I antigen by exon shuffling of *H-2D^d* and a gene (*Q10^b*) derived from the *Qa* region (20). However, for the *in vivo* studies described here we preferred to use a soluble class I antigen containing all three outer domains of a classical transplantation antigen.

We have established transgenic mice that integrated several copies of the modified *H-2K*^{k} gene in their genome. Several independent methods showed that the transgene was expressed and that different cell types could secrete the H-2K^k protein. Soluble H-2K^k molecules were found at serum concentrations up to 200 ng/ml.

First, the influence of soluble self H-2K^k on the CTL precursor and B-cell populations reactive against surface-bound H-2K^k was investigated. Therefore, for immunization spleen cells were chosen that carried additional helper determinants (e.g., Ia^k). The transgenic animals could generate a normal anti-H-2K^k CTL response as measured in bulk cultures and by limiting dilution analysis. The soluble H-2K^k is expressed in the thymus, where, in general, T-cell tolerance for surface-bound self MHC antigens is induced. Therefore, the data suggest that soluble H-2K^k would not act as a tolerogen for CTL precursors, possibly because cross-linking of T-cell receptors and/or interaction of other cell-surface molecules is required for tolerance induction. Similar data were obtained when CTL were raised against Q10 antigen determinants expressed on a hybrid antigen on the cell surface of stimulator cells in mice, which had ≈30 μg of the soluble nonclassical transplantation antigen Q10 per ml of serum (21).

The transgenic animals could also produce antibodies against membrane-associated H-2K^k in spite of the presence of soluble H-2K^k in these mice. The ability to mount CTL and antibody responses was not due to late expression of the soluble antigen during ontogeny. Transgenic embryos expressed soluble H-2K^k on day 18 or 19 of gestation. At this time in development, injection of allogeneic cells of a certain haplotype induced tolerance toward a skin graft of the same haplotype in adult life (22).

Natural tolerance to protein antigens depends on the amount of protein present in the serum, which can vary from 25 ng/ml to 10 μg/ml depending on the antigenic system (23, 24). The question can therefore not definitely be answered if the amount of 200 ng/ml of soluble H-2K^k in the serum is too low to induce tolerance. It should also be noted that since soluble H-2K^k is produced in the thymus the amount of this material in the thymic microenvironment could be quite different from the amount circulating in the bloodstream.

So far we have shown that CTL precursors and B cells specific for membrane-associated H-2K^k could not be made tolerant by soluble H-2K^k in the transgenic mice. In most of the reported cases, natural tolerance to protein antigens was based on tolerance in the T-helper-cell compartment (24, 25). Since T-helper cells have not yet been tested in our system, our data do not exclude the possibility that the soluble H-2K^k has induced tolerance in specific T-helper cells. In this context, previous reports should be noted showing the existence of T-helper cells recognizing alloantigen in their native form (unrestricted recognition) and other T-helper cells that see alloantigen in a processed form as indicated by the MHC restricted recognition (26, 27).

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