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*J Immunol* 1998; 160:674-680;
http://www.jimmunol.org/content/160/2/674

This information is current as of April 16, 2017.
NK Cells from Human MHC Class I (HLA-B) Transgenic Mice Do Not Mediate Hybrid Resistance Killing Against Parental Nontransgenic cells

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We have investigated the capacity of human MHC class I HLA-B gene products, HLA-B27, -B7 (fully human), and -B7Kb (human-mouse hybrid consisting of the α1 and α2 domains of HLA-B7, and the α3 and cytoplasmic domains of mouse H-2Kb), expressed on mouse NK cells during ontogeny to influence NK recognition of otherwise syngeneic mouse target cells. Despite a high level of surface expression of the transgene (comparable to that of endogeneous H-2DβKb molecules), the direct killing of YAC-1 targets, and the killing of P815 targets in a redirected lysis assay, the NK effectors of these transgenic mice could not mediate hybrid resistance-like killing of nontransgenic C57BL/6 target cells either in vitro or in vivo. Splenocytes from B6-B27 mice could be used to generate CTL lines against a B27-binding peptide, implying that T cells restricted by HLA-B27 developed during ontogeny. NK cells from B6-B27 could lyse B6-B27 Con A lymphoblasts pulsed with Dβ-binding peptide but not B27-binding peptides. Taken together, our results show that these human HLA-B transgenic products cannot function as class I MHC "self" elements for mouse NK cells, even when present throughout ontogeny. The Journal of Immunology, 1998, 160: 674–680.

N atural killer cells are a small population of bone marrow-derived lymphocytes that can kill a broad range of target cells, including some tumor cells and virus-infected cells, and also bone marrow cell allografts in irradiated mice (1–4). It is now generally accepted that NK cells recognize and kill target cells through the interplay of activating and inhibitory receptors (5–7), and that the activating signal from the activating receptor(s) can be overridden by a dominant negative signal from an inhibitory receptor if the inhibitory receptor(s) is interacting with its ligand(s) on the target cells at the same time. The ligand for some (perhaps all) inhibitory receptors is associated with class I MHC, thus explaining why lack of expression of self-MHC molecules results in susceptibility to NK cell-mediated lysis ("missing self" hypothesis; 8). The prototypic murine inhibitory receptor, the Ly49A receptor for H-2D and D molecules, is a type II transmembrane protein characterized by a C-type lectin domain (9, 10). In humans, two surface glycoproteins (p58) have been shown to be inhibitory receptors for certain HLA-C alleles; a 70-kDa glycoprotein (p70 or NKB1) is the inhibitory receptor for HLA-Bα1 al-

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Received for publication July 22, 1997. Accepted for publication October 1, 1997.

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1 This work was supported by the Connaught Laboratories/University of Toronto Research Fund (R.G.M.); S.K.K. is a research student of the National Cancer Institute of Canada and was supported with funds provided by the Canadian Cancer Society.

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Abbreviations used in this paper: HLA-B, human leukocyte antigen B genes; B6-B27, B6 mice carrying HLA-B27 transgenes; B6-B7, B6 mice carrying HLA-B7 transgenes; B6-B7Kb, B6 mice carrying a hybrid genomic clone encoding the α1 and α2 domains of HLA-B7, and the α3 and cytoplasmic domains of mouse H-2Kb; CM, complete medium; LAK, lymphokine-activated killer; OVAp, OVA peptide.
involvement of MHC-bound peptides in MHC recognition by murine and human NK cells (17, 25–27).

In the present study, we have evaluated the capacity of human HLA-B gene products expressed on mouse NK cells during ontogeny to influence NK recognition of otherwise syngeneic mouse target cells.

Materials and Methods

Animals

C57BL/6 (B6, H-2b), BALB/c (H-2d), and (BALB/c × B6) F1 (CByB6F1, H-2M2) were purchased from The Jackson Laboratory, Bar Harbor, ME. Mice were kept in a specific pathogen-free environment. In most experiments, 6- to 10-wk-old female mice were used (although either sex gave similar results). The HLA transgenic mice have been described (Ref. 28, and manuscript in preparation), and were as follows: HLA-B7 mice carried a fully human genomic B7 clone; HLA-B27 mice carried a fully human genomic B2705 clone; B7/Kb mice carried a hybrid genomic clone encoding the α1 and α2 domains of HLA-B7, and the α3 and cytoplasmic domains of mouse H-2Kb (28). The first two transgenic mice also carried human β2-microglobulin genes. All transgenic mice used here have been backcrossed to B6 mice four or more generations in the animal colony of the Ontario Cancer Institute.

Poly(I:C) treatment

To boost the in vivo NK activity of animals (29), poly(I:C) (St. Louis, MO) in sterile PBS (100 μg) was i.p. injected into mice on days 0 and 1. Mice were killed for experimentation on day 3.

mAbs and flow cytometry

Hybridomas PK136/HB191 (anti-NK1.1), HB51 (anti-KbD), and HB119 (anti-NK1.1), and PK136/HB119 (anti-NK1.1) were established in our laboratory. Culture supernatants were purified by protein A (Sigma) chromatography. Purified mAbs were biotinylated and then labeled with CyChrome (Molecular Probes, Eugene, OR) and used as primary antibodies to detect NK cells by flow cytometry. 

Preparation of splenocytes

Spleens were pressed through a wire mesh screen with a disposable syringe plunger into complete medium (CM) which was α-MEM (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% FCS (Life Technologies), 50 μM 2-ME, and 10 mM HEPES. Released cells were layered over 5 ml of 6% BSA in PBS, counted, and resuspended in PBS. A total of 3 × 10⁶ cells were incubated with a FITC solution (30 μg/ml PBS final; Sigma) at 37°C for 18 min. Excess FITC was removed by centrifuging the cells through 3 ml of 6% BSA/PBS. The cells were washed twice with 1% BSA/PBS, counted, and resuspended in PBS. A total of 3 × 10⁶ FITC-labeled cells of B6 or BALB/c origin (in 0.3 ml PBS) were injected into the lateral tail vein of the recipient mice. 

Peptide-prepulsed lymphoblast targets in NK cytotoxicity assay

Activated NK cells were produced by culturing ~10 × 10⁶ nylon wool nonadherent spleen cells from HLA-B27 transgenic mice in 5 ml of CM containing 500 μl/ml of mouse IL-2 as described previously. Target cells were HLA-B27 + Con A blasts from the HLA-B27 transgenic mice. These target cells were 3Cr-labeled, washed, and incubated with or without HLA-B27-specific optimal peptides (BAY9KSTEII, KRFEGILTQR) (33). A 3Cr-binding optimal peptide (Flu-NP, ASHENMETM) was used as a positive control (34). Target cells were incubated with 100 ng/ml of peptides in 3 ml of CM for 45 min at 4°C and washed twice before being used in a 4.5-h 3Cr release assay as described above.

Generation and maintenance of a peptide-specific CTL

Generation of peptide-specific CTL was performed as described previously (35). Briefly, spleen cells from HLA-B27 transgenic mice were depleted of B cells by passage through nylon wool and cultured at 5 × 10⁶ cells/ml in 10 ml of CM in the presence of 1 ng/ml of B27-specific peptide (KRFEGILTQR) and 5 μl/ml of mouse IL-2. On day 7, CTL were harvested on lymphocyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) and used in the cytotoxicity assay. Short-term maintenance of the CTL line was performed by culturing ~2 to 3 × 10⁶ cells with 5 μl/ml of mouse IL-2. An OVA peptide (OVA-specific CTL line was generated as described by using spleen cells from B6 mice and OVA265-279 peptide (SIINFEKL) (36).

Results

Surface expression of H-2DKKb and HLA-B molecules in the transgenic mice is comparable

Splenocytes from nontransgenic B6 and CByB6F1 mice, and B6 transgenic mice carrying HLA-B7, HLA-B7Kb, and HLA-B27 transgenes (abbreviated as B6-B7, B6-B7Kb, and B6-B27, respectively) were stained for surface expression of endogeneous H-2DKKb and transgenic HLA molecules. As shown in Figure 1, B6 mice expressed an approximately twofold higher level of H-2DKKb molecules than a nontransgenic F1 mouse as expected, whereas the transgenic mice expressed H-2DKKb molecules at a level comparable to that of a B6 mouse (and higher than that of an F1 mouse).
splenocytes of B6, F1, B6-B7, and B6-B7Kb mice. It was determined that naive B6 and F1 splenocytes, respectively, carried approximately $4 \times 10^5$ and $2 \times 10^5$ binding sites for H-2Dk molecules, and that both HLA-B transgene products were expressed at a level comparable to that of H-2Dk molecules on F1 splenocytes (~1 to $3 \times 10^5$ binding sites) (data not shown).

NK cells from HLA transgenic mice do not mediate hybrid resistance

LAK cells were prepared from splenocytes of the F1, B6, and HLA-B transgenic mice (B6-B7, B6-B7Kb, and B6-B27). They were shown to be fully functional in an Ab-mediated redirected lysis assay (Fig. 2A). In this assay, a mAb reactive with an NK-activating receptor (here NK1.1 or 2B4) binds specifically to the effector NK cell while its Fc portion binds to a target cell Fc receptor (here P815) that provides a bridging and cross-linking effect. The LAK also exhibited comparable YAC-1 killing activity (Fig. 2B). However, LAK effectors from B6-B7, B6-B7Kb, and B6-B27 mice did not recognize B6 Con A lymphoblasts as targets in an in vitro hybrid resistance assay. As illustrated in Figure 2B in which all the effectors (B6-B7, B6-B7Kb, B6-B27, B6, and F1) were assayed for their anti-B6 target activity in one experiment under identical conditions, the LAK cells of the B6-B7, B6-B7Kb, and B6-B27 mice, as well as the LAK cells of B6 mice, all showed low or no killing of B6 Con A targets (<15%), compared with the ~25% B6 Con A target lysis observed in the hybrid resistance setting (in which F1 LAK cells were used) in the same experiment. In four other separate experiments in which LAK cells from different HLA-B transgenic mice were compared with LAK cells from F1 mice for the ability to recognize B6 targets in vitro, none of the HLA-B transgenic mice showed significant killing of the B6 Con A targets, whereas the F1 anti-B6 lysis was always significant, varying from 25 to 37%.

These in vitro findings were further confirmed by the analysis of hybrid resistance-like killing in vivo. It has been shown that FITC-labeled B6 lymphoid cells persist in a syngeneic host but not in a CByB6F1 hybrid mouse (hybrid resistance in vivo) (21, 29, Fig.

FIGURE 1. Surface expression of H-2Dk and HLA-B molecules in the transgenic mice. Splenocytes from naive B6 and B6 mice carrying HLA-B7, HLA-B7Kb, and HLA-B27 transgenes, as well as CByB6F1 mice were stained for surface expression of endogenous H-2Dk and the HLA-B transgenes by biotin-conjugated mAbs HB51 (anti-H-2Dk) and ME-1 (anti-HLA-B7 and -B27) respectively, followed by streptavidin-phycocerythrin. The fluorescence and light-scatter properties of individual lymphocytes were measured and analyzed on a FACScan flow cytometer (Becton Dickinson).
We therefore went on to test whether FITC-labeled B6 lymphoid cells could persist in the HLA-B transgenic animals in vivo in this previously described and validated flow cytometric assay of hybrid resistance. As shown in Figure 3, the FITC-labeled cells persisted in all the transgenic mice, as well as in syngeneic B6 mice. In addition, the injected FITC-labeled B6 donor cells were found to persist in the transgenic mice even when the NK activity of the host animals was boosted by poly(I:C) treatment (Fig. 3).

A CTL line can be generated against a B27-binding peptide
Using splenocytes from B6-B27 mice, we used a B27-specific peptide (KRFEGLTQR) to raise a peptide-specific CTL line. As shown in Figure 4, B6-B27 lymphoblasts pulsed with the KRFEGLTQR peptide (but not RRYQKSTEL peptide, another B27-specific peptide) were lysed by the CTL line, confirming that the peptide could bind to B27 and form a specific CTL target structure. In fact, the ability of the KRFEGLTQR peptide to form a CTL target structure on the B6-B27 lymphoblasts was comparable to that of the Kb-specific optimal peptide (OVAp) when OVAp-specific CTL and OVAp-pulsed B6 lymphoblasts were used.

Pulsing HLA-B27 Con A lymphoblast targets with B27-binding peptides does not induce NK-mediated lysis
It has been shown that normal B6 lymphoblasts become more sensitive to lysis by NK cells after being incubated with peptide that can bind to their MHC class I molecules (17, 27). Here, we examined whether pulsing B6-B27 Con A lymphoblasts with B27-specific optimal peptides (which were identified by peptide elution from HLA-B27) (33) can induce NK cells of B6-B27 transgenic...
mice to kill the otherwise syngeneic targets. As a control, we have pulsed the same B6-B27 Con A blast cells from the B6-B27 transgenic mice and B6 Con A blast cells for B27 peptide-specific and OVAp-specific CTLs, respectively. These target cells were 51Cr labeled, washed, and pulsed with or without 100 ng/ml of peptide for 45 min at 4°C and washed twice before being used in a 4.5-h 51Cr release assay. KRFEGLTQR-specific and the OVAp-specific CTL effectors were mixed with the corresponding 51Cr-labeled targets at 30:1 and 50:1 E:T ratios, respectively. Control peptides used in the experiment were B27-binding peptide (RRYQKSTEL) and a D8-binding optimal peptide (Flu-NP, ASNENMETM) for the KRFEGLTQR-specific CTL and the OVAp-specific CTL, respectively.

Discussion

We have investigated the capacity of human HLA-B27 and HLA-B7 transgene products expressed on B6 mouse NK cells during ontogeny to influence NK recognition of otherwise syngeneic B6 mouse target cells. The HLA transgene products were expressed at the cell surface at a level comparable to the endogenous H-2Db Kb molecules (Fig. 1). In agreement with previous studies, we found that pulsing the B6-B27 lymphoblasts with Flu-NP peptide induced the lysis of the target cells (Fig. 5). However, we did not observe such an enhancement of lysis when either HLA-B27-specific peptide (RRYQKSTEL, KRFEGLTQR) was used in the assay (Fig. 5). Taken together, we have shown that pulsing B6-B27 Con A lymphoblast targets with B27-binding peptides does not induce NK-mediated lysis despite the fact that the B27-specific peptide can bind to the HLA-B27 and form a CTL target structure.
Although the HLA transgenes (HLA-B27 and HLA-B7) used here have been previously shown to confer protection from human NK cell lysis, they could not “educate” or shape the NK specificity of the NK cells developed in these transgenic animals (Fig. 2, 5). It has been shown that transfection of certain HLA alleles can result in loss of susceptibility to human NK-mediated lysis (23, 24), and that as little as a twofold down-regulation of class I MHC can double the amount of lysis observed for a particular target cell (38). However, we argued against the possibility that the lack of “education” was simply due to a relatively low-level expression of the HLA transgenes in the transgenic mice, because surface expression of the HLA-B transgene products was found to be comparable to that of the H-2D<sup>K</sup><sup>b</sup> molecules on normal F<sub>1</sub> splenocytes. Also, we do not favor the notion that the lack of “education” is due to a species-specific difference dependent on α3 or the cytoplasmic tail of human and mouse MHC class I molecules, because we did not see any significant B6 Con A blast lysis even in mice carrying the HLA-B7K<sup>b</sup> (α1 and α2 domains of HLA-B7, α3 and cytoplasmic domains of mouse H-2K<sup>β</sup>) transgene. In fact, this finding further suggests that a critical interaction between the NK receptor(s) and its ligands involves the α1 and α2 domains of MHC class I molecules. Cells resistant to lysis by NK cells can become sensitive to lysis if peptides are added that can bind to their class I MHC (17, 27, 39). Storkus et al. (39) have shown that binding of peptide to transfected HLA class I molecules (which was protective against human NK killing) restored the susceptibility to lysis. Chadwick and Miller (17) and Chadwick et al. (27) found that normal, untransformed mouse lymphoblasts, resistant to lysis by syngeneic mouse NK cells, became sensitive to lysis if incubated with peptides that could bind to the class I of the target, and Su et al. have shown that peptide binding is destroying an inhibitory ligand(s) for the putative NK receptor(s) that confers protection to the syngenic target cells. However, when we used two optimal HLA-B27-binding peptides in this system, we did not see an augmentation of the killing response (Fig. 5).

The lack of “education” in all of the HLA-B transgenic mice, together with the finding that peptide prepping of a B6-B27 target could not sensitize it to be lysed by B6-B27 transgenic NK LAK suggest that at least these human class I MHC alleles are inert structures in terms of mouse NK ontogeny and recognition. There are several possible explanations. It may be that we were unfortunate in our choices of human class I MHC molecules tested and that additional choices would yield human class I MHC molecules that would function in mouse NK ontogeny and recognition. Alternatively, mouse germ line NK receptors may be inherently, structurally incapable of recognizing human class I MHC, and this capacity cannot be developed during ontogeny. Note that in the D8 transgenic mouse (B6 mouse with D<sup>α</sup> transgene), introduction of the H-2D<sup>α</sup> transgene into B6 mice reduced Ly49A expression by 30 to 50%. Ly49A is known to bind to D<sup>α</sup> molecules and deliver a negative signal to NK cells upon recognizing D<sup>α</sup> molecules (9). It is hypothesized that the result of such Ly49A receptor calibration (by its ligand, D<sup>α</sup> transgene products in the D8 mice) leads to a change in the NK target specificities in which the Ly49A receptor from D<sup>α</sup> mice acquire the ability to kill previously resistant B6 Con A blasts, and the low H-2D<sup>α</sup> target SP2/0 (40). Here, in our HLA-B27 transgenic mice, the inability of murine NK receptors to interact with human HLA-B transgene products might lead to failures in receptor(s) calibration, as well as alteration of NK killing phenotypes. In humans, Ig superfamily (p58, p70, and p140) and lectin-like killer inhibitory receptors (CD94/NKG2A heterodimers) have

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been found, whereas in the mouse, only (different) lectin-like inhibitory receptors (Ly49 family members) have been identified, consistent with the possibility that human and murine inhibitory receptors use distinct strategies for selection of NK cells.

Finally, a quite different explanation for the failure of human class I MHC molecules to function in mouse NK ontogeny and recognition is that there may be an auxiliary molecule involved in NK ontogeny and/or in recognition of class I MHC. This interaction is species specific such that the murine auxiliary molecule cannot interact with human class I MHC. This would be analogous to the β2-microglobulin molecule in which mouse β2-microglobulin cannot bind effectively to human class I MHC heavy chains so that cell surface expression is low unless human β2-microglobulin is also provided.

Acknowledgments

We thank Dr. H. Karasuayama (University of Tokyo, Tokyo, Japan) for the gift of a mouse Hλ-2 transfectant cell line.

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