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Abnormal class I assembly and peptide presentation in the nonobese diabetic mouse

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ABSTRACT **Presentation of self-antigens by major histocompatibility complex (MHC) class I molecules requires the function of the MHC class II-linked genes *Tap-1* and *Tap-2*. Evidence suggests that interruption of self-peptide presentation results in reduced cell surface expression of MHC class I molecules and the interruption correlates with progression to diabetic autoimmunity in nonobese diabetic (NOD) mice and humans. NOD mice possess a rare *Tap-1* allele (*Tap-1^b*); this is associated with reduced Tap-1 mRNA abundance in lymphocytes from diabetes-prone females and decreased conformationally correct class I molecules on the cell surface. In this study, we demonstrate that, similar to lymphoma cell lines with mutations in *Tap-1* or *Tap-2*, the reduced expression of class I molecules on the surface of lymphocytes from diabetes-prone female NOD mice was normalized by incubation at low temperatures or by exposure to class I allele-specific peptides. As would be expected for cells that express surface class I molecules not associated with peptide, female NOD lymphocytes were resistant to lysis by class I-restricted, peptide-specific cytotoxic T lymphocytes. Furthermore, the rate of class I exit from the endoplasmic reticulum of lymphocytes from female NOD mice was delayed as demonstrated by delayed glycosylation. Male NOD mice, which are not prone to diabetes, lacked these functional defects in class I assembly and had near-normal levels of Tap-1 mRNA and exhibited normal density of class I epitopes that were peptide filled. These results are consistent with the possibility that the rare *Tap-1^b* allele is associated with a quantitative defect in *Tap-1* expression that influences disease course.**

CD8$^+$ T cells recognize short peptides bound in the outwardly facing groove of major histocompatibility complex (MHC) class I molecules on the surface of cells; recent studies have shown that the peptides in the groove of class I molecules are fragments of endogenous antigens (1–5). Genetic evidence indicates that the delivery of these peptide fragments from the cytosol into the endoplasmic reticulum is controlled by two MHC class II-linked genes, *Tap-1* and *Tap-2* (6–15). The assembly of class I molecules in the endoplasmic reticulum for subsequent egress to the cell surface requires, in addition to intact class I molecules, allele-specific peptide delivery by the Tap-1–Tap-2 heterodimer. The complex of an endogenous peptide fragment and class I on the cell surface is critical for T-cell education and tolerance to self.

The identification of the critical function of Tap-1 and Tap-2 in antigen presentation by class I molecules was secondary to the isolation of mutant lymphoblastoid cell lines—such as murine RMA-S cells and human BM36.1, LBL 721.174, and derivative T2 cell lines—selected for low cell surface expression of class I molecules (8, 16–18). Genetic studies subsequently identified either point mutations or deletions of *Tap-1* or *Tap-2* in these cell lines (2, 6, 7, 15). The surface expression of class I molecules on these mutant cell lines is not only reduced, especially as revealed with conformation-dependent anti-class I antibodies, but also unstable. Surface class I expression can be stabilized by low temperatures, which reduce the rate of turnover of surface class I molecules. The mutant cell lines also show delayed egress of class I molecules from the endoplasmic reticulum and are resistant to cytotoxic T-lymphocyte (CTL) lysis (2, 11, 19). Most of these functional abnormalities in the mutant cells can be corrected by "peptide feeding" (20–22). Low-temperature culture increases the surface density of empty class I but does not restore self-peptide presentation (20, 22).

We have recently described faulty antigen presentation by class I molecules in humans with insulin-dependent diabetes mellitus and in the murine nonobese diabetic (NOD) model of diabetes (23). In diabetes-prone female NOD mice, low Tap-1 (formerly Ham-1 in the mouse) mRNA abundance correlates with low lymphocyte surface expression of conformationally correct class I molecules and disease penetrance (24). The NOD mouse has a rare *Tap-1* (*Tap-1^b*) allele but a common *Tap-2* allele, which is associated with near-normal Tap-2 mRNA abundance in diabetes-prone females (23).

To investigate whether the low class I expression on antigen-presenting cells of diabetes-prone female NOD mice is associated with faulty loading of the class I molecules with endogenous peptides, we examined NOD lymphocytes for the functional defects in class I assembly and antigen presentation associated with altered function of the class II-linked ATPase transporters that have been described in the transporter literature over the past 3 years.

MATERIALS AND METHODS

Fluorescence-Activated Cell Analysis. Spleen cells isolated from NOD (K^d, D^b), C57BL/6 (K^b, D^b), and BALB/c (K^d, D^d) mice were incubated with fluorescein-conjugated anti-K^d antibody 31-3-45 or fluorescein-conjugated anti-D^b antibody H141-31 [American Type Culture Collection (ATCC)]. In addition, H-2 class I antibody M1/42 (ATCC) was also used. The fluorescence-activated cell sorting (FACS) analysis gate was set for either monocytes/macrophages or lymphocytes by size with forward light scatter versus log(fluorescence). The monocyte/macrophage peak was verified by a macrophage-specific antibody, and the lymphocyte peak was verified with antibodies to CD4 and CD8.

CTL Analysis. Virus-specific CTLs were generated as bulk CTLs isolated from splenocytes by *in vivo* followed by *in vitro* priming of BALB/c (K^d, D^d), B10.A(2R) (K^k, D^b), D2.GD (K^d, D^b), or C57BL/6 (K^b, D^b) mice with live influenza virus A2/Japan/305/57. Mice were injected intraperitoneally with 0.3 ml of a virus stock solution (1 ml of viral stock solution contains 10^6–3.16×10^8 tissue culture 50% infective dose) in phosphate-buffered saline. Two to 3 weeks

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Abbreviations: MHC, major histocompatibility complex; NOD, nonobese diabetic; FACS, fluorescence-activated cell sorting; CTL, cytotoxic T lymphocyte; ATCC, American Type Culture Collection.
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later, the animals were injected with a further 0.15 ml of virus stock solution. Two days later, the animals were killed, and splenocytes were prepared ($1 \times 10^6 - 5 \times 10^6$ cells per ml) and cultured in RPMI containing 10% (vol/vol) fetal bovine serum and live virus stock solution. After 5–6 days in culture, the splenocytes were centrifuged over Ficoll (Pharmacia) to obtain viable CTLs, which were resuspended in medium at the appropriate effector-to-target ratios for the cytotoxicity assay (1, 25, 26). Target lymphocytes were prepared from lipopolysaccharide-stimulated splenocytes. Splenocytes harvested from female mice were cultured for 2–3 days in RPMI with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and lipopolysaccharide (40 μg/ml·Difco). Cells (10^7/ml) were then incubated with ^{51}Cr (0.1 mCi/ml; 1 Ci = 37 GBq) at 37°C for 1 hr. After three gentle washes, the ^{51}Cr-labeled targets were then incubated with live virus (100 μl/ml) for 60 min at 37°C. Alternatively, the targets were incubated at this step with control medium (no virus). The target cells were then plated at a concentration of 10^5 cells per well with the appropriate number of CTL effectors to generate the described effector-to-target ratios. CTL clones 14.1 and 11.1 are CTLs specific for hemagglutinin from A/JAP of the H2N2 subtype (1). All are expressed as percent specific lysis, and data represents means for triplicates. For experiments related to the addition of exogenous class I-specific susceptibility of female NOD target cell to CTL lysis, the targets were incubated either with live A/PR influenza virus or with synthetic A/PR influenza viral peptide fragment (+) Thr-Tyr-Gln-Arg-Thr-Arg-Ala-Leu-Val (5 μg/ml). Bulk K^d-restricted, A/PR virus-specific CTLs were prepared from BALB/c mice and showed no cytotoxicity against irrelevant virus-infected targets, C57BL/6 ($H\text{-}2D^b$), or against relevant (BALB/c) targets not exposed to virus. Spontaneous release for all targets ranged from 5% to 17%.

Pulse–Chase Experiments. Splenocytes were incubated at 37°C for 90 min in methionine-free medium and then labeled with [^{35}S]methionine (10 mCi/ml) for 15–20 min. The [^{35}S]methionine medium was removed, and the cells were washed once and resuspended in medium containing 16.7 mM unlabeled methionine. Cells (5×10^6 or 1×10^7) were subsequently harvested at various times, lysed with 0.5–1.0 ml of 1% lysis buffer, and subjected to immunoprecipitation with a conformational-independent antibody to K^d (clone 28-14-8S; ATCC).

mRNA Production. A single spleen was excised from B10.D2 (R103), BALB/c, or NOD mice and immediately immersed into lysis buffer for rapid homogenization by a homogenizer. To accurately quantitate *in vivo* mRNA levels, the spleens were never exposed to medium or fetal calf serum. The splenic mRNA was prepared using InVitrogen FastTrack mRNA isolation system (San Diego). Approximately 0.5–1.0 μg of highly pure mRNA was run in a 1% agarose gel and then transblotted to GeneScreen*Plus* hybridization membranes (DuPont).

RESULTS

MHC Class I Expression in Male and Female NOD Mice. Similar to many autoimmune diseases in humans, diabetes in NOD mice develops predominantly in females. In our colony, <3% of males develop hyperglycemia by 6 months of age, compared to 86% of females. As has been demonstrated previously (23), MHC class I density of peptide-filled class I on female NOD lymphocytes is depressed when examined with conformationally dependent antibodies directed to K^d (clone 31-3-45) and D^b (clone H141-31) (Table 1). Furthermore, all subpopulations of lymphocytes from female NOD mice that progress to hyperglycemia exhibit a reduced surface density (fluorescence) of conformationally dependent class I molecules for both alleles of the class I genes K^d and D^b, as revealed by FACS analysis (Fig. 1). An expansion of this study to non-diabetes-prone male NOD mice revealed that both lymphocytes and monocytes at 8–11 weeks of age showed normal levels of conformationally correct class I proteins. The density of peptide-loaded class I on the surface of male NOD mice was indistinguishable from matched control mice when analyzed with conformationally dependent class I antibodies directed to K^d or to D^b (Table 1).

Table 1. Surface density of conformationally correct class I molecules

Strain	Sex	Lymphocytes *n*	Lymphocytes Mean	Lymphocytes *P*	Macrophages *n*	Macrophages Mean	Macrophages *P*
			Anti-H-2K^d antibody				
NOD	F	21	3.0 ± 0.74	<0.001	21	17 ± 9.2	<0.001
BALB/c	F		5.0 ± 1.7			33 ± 15	
NOD	M	11	4.8 ± 1.5	0.14	20	37 ± 22	0.50
BALB/c	M		5.4 ± 1.6			34 ± 15	
			Anti-H-2D^b antibody				
NOD	F	11	5.2 ± 2.7	0.005	10	20 ± 8.2	0.012
C57BL/6	F		7.9 ± 2.1			32 ± 7.9	
NOD	M	10	7.3 ± 5.5	0.52	19	36 ± 23	0.35
C57BL/6	M		8.0 ± 3.3			32 ± 14	

Spleen cells isolated from NOD, C57BL/6, and BALB/c mice were incubated with fluorescein-conjugated anti-H-2K^d antibody or fluorescein-conjugated anti-H-2D^b antibody and analyzed by FACS. The values given are the mean fluorescence ± SD. *n*, Number of paired samples; *P*, *P* value compared to the control (BALB/c or C57BL/6).

Stabilization of Class I by Low-Temperature Culture. Class I expression can be stabilized in transporter-mutant RMA-S cells by incubation at reduced temperatures (20, 22). We therefore incubated splenocytes from diabetes-prone female NOD mice at reduced temperatures in an attempt to decrease the suspected accelerated rate of turnover of the empty K^d or D^b molecules. Female NOD mouse lymphocytes cultured at 28°C for >18 hr demonstrated increased class I expression as revealed by staining with an anti-K^d antibody with a mean log(fluorescence) increase of 80%. This represents a value not statistically different from controls. In addition, culture of NOD female splenocytes at temperatures of 25°C, 28°C, and

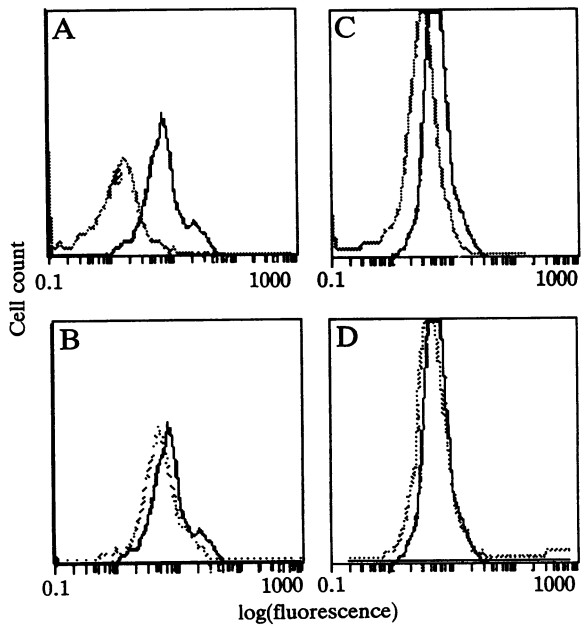

FIG. 1. FACS analysis of surface K^d (*A* and *B*) and D^b (*C* and *D*) class I expression on lymphocytes from NOD female (*A* and *C*) and male (*B* and *D*) mice (dashed line) compared to lymphocytes from control strains (solid line).

37°C for 24 hr demonstrated that normalization of K^d and D^b expression occurred on macrophages only at reduced temperatures of 25°C and 28°C but not at physiologic temperatures of 37°C.

Correction of NOD Low Class I with Exogenous Peptide Feeding. Enhanced stability of class I expression on the surface of transporter-mutant cell lines can also be achieved by treatment of cells with synthetic peptides specific for the class I allele. We therefore cultured female NOD splenocytes with a K^d allele-specific peptide in an attempt to load the empty peptide groove of this class I molecule. The peptide corresponding to amino acids 147–155 of the influenza virus A/PR nucleoprotein is presented in the H-2K^d groove to H-2-restricted CTLs (5). A synthetic peptide corresponding to this amino acid sequence, at a concentration of 480 μM, stabilized the surface expression of K^d in female NOD mouse macrophages over the course of a 5-hr incubation at 37°C (Fig. 2). Incubation of this peptide with control class I-identical B10.D2 (R103) splenocytes resulted in no change in mean fluorescence attributable to K^d. The restoration and stabilization of K^d expression on female NOD splenocytes was not only allele and peptide specific but dose dependent.

Delayed Egress of Class I from the Endoplasmic Reticulum of NOD Female Mice. The rate of intracellular transport of class I molecules from the endoplasmic reticulum to the Golgi can be estimated by the rate of sialylation of these molecules, which occurs in the Golgi and results in an increase in their molecular mass as revealed by SDS/PAGE. An insufficient supply or correct type of endogenous peptide transport by disruption of Tap-1 or Tap-2 results in delayed class I egress due to faulty class I assembly. In [^{35}S]methionine pulse–chase experiments, immunoprecipitates from control mouse strain splenocytes or male NOD mouse splenocytes, prepared with an antibody to K^d, revealed similar rates of class I glycan modification (Fig. 3), suggesting a normal rate of class I egress. In contrast, female NOD splenocytes exhibited delayed delivery of class I molecules into the trans-Golgi as evidenced by a delay in achieving a higher molecular weight class I mass (Fig. 3), presumably indicative of failed assembly of the trimer complex of class I molecule, self-peptide, and β_2-microglobulin in the endoplasmic reticulum. At 15 min of the pulse–chase, initial levels of sialylation of class I was visible in both the NOD male and B10.D2 (R103) control

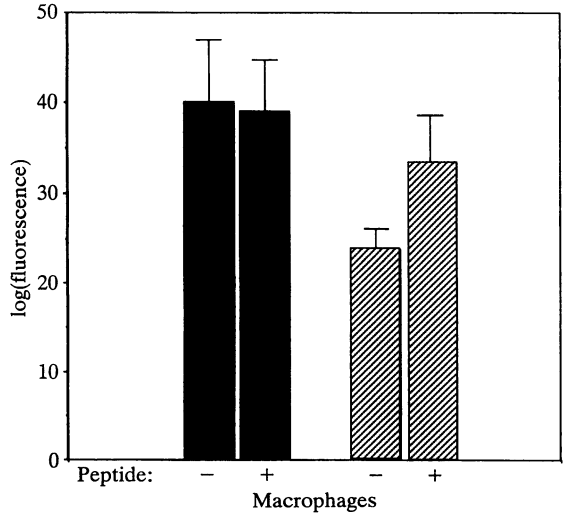

FIG. 2. Effect of exogenous synthetic peptide on macrophage surface expression of class I molecules. Control B10.D2 (R103) splenocytes (solid bars) or NOD splenocytes (hatched bars) from females were cultured in serum-containing medium at 37°C with (+) or without (−) K^d-specific peptide (480 μM) for 5 hr and then analyzed by FACS with anti-K^d antibody.

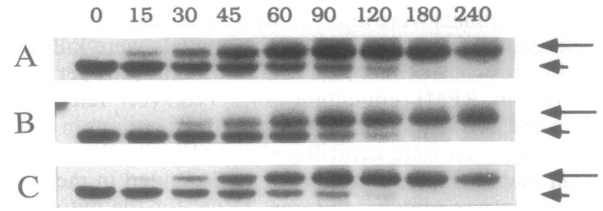

FIG. 3. Endoplasmic reticulum transport of class I molecules in female NOD mouse splenocytes (*B*) compared to male NOD splenocytes (*C*) and control mouse B10.D2 (R103) splenocytes (*A*). The positions of sialylated and nonsialylated K^d are indicated by arrows and arrowheads, respectively. The values along the top of the gel indicate the number of minutes.

mouse; the female NOD class I demonstrated no sialylation at this early time point. Similarly, at 45 min of the pulse–chase, 50% sialylation of class I was visible in only the NOD male and control splenocytes: the female NOD class I exit continued to be slowed.

Decreased Susceptibility of NOD Female Lymphocytes to Peptide-Specific CTLs. Transporter-mutant cells with defects in *Tap-1* or *Tap-2* are incapable of presenting internally derived cytoplasmic antigens in the context of class I molecules and therefore are not susceptible to peptide- and class I-restricted CTL lysis. This is a sensitive and specific test of normal class I presentation. We therefore tested the ability of female NOD lymphocytes to serve as targets for lysis by K^d- or D^b-restricted influenza virus (A/JAP)-specific CTL clones or bulk CTLs specific for a specific class I allele and virus. Both D^b-restricted and K^d-restricted bulk CTLs showed negligible or no killing of virus-exposed NOD female targets in over 30 separate CTL experiments (Table 2; partial data shown). Normal CTL lysis of class I-matched targets from class I-matched control mice pulsed with live virus was observed for many different murine strains. In addition, both the K^d-restricted CTL clone 14-1 and CTL clone 11-1 specific for hemagglutinin from A/JAP failed to lyse female NOD target lymphocytes (Table 2; data for clone 14-1 shown). The resistance of female NOD lymphocytes to sensitive class I-restricted peptide-specific CTLs suggests that, similar to mutant RMA-S and LCL 721.174 cells, peptide-loaded class I molecules are inadequately or incorrectly present on the surface of these cells.

It has been demonstrated that the class I molecules exported to the cell surface at reduced temperatures in mutant RMA-S cells lack peptide in their antigen-binding pocket. To determine if this is also true of female NOD mouse lymphocytes, we incubated the cells at 28°C for 24 hr and then used them as targets for K^d- or D^b-restricted influenza virus-specific CTLs. The phenotypic correction of class I expression on female NOD targets by low-temperature culture did not restore susceptibility to specific CTL lysis (data not shown). This result suggests that the increased class I expression on the surface of female NOD lymphocytes at 28°C is attributable to stability of the empty class I molecules. In contrast, the phenotypic correction of class I expression on female NOD lymphocytes achieved by incubation with class I-specific peptide did restore susceptibility to CTLs (Table 3). Neither the susceptibility of control BALB/c targets nor that of NOD male targets to CTL lysis was affected by prior incubation with K^d-specific peptide (Table 3).

Normal Susceptibility of NOD Male Lymphocytes to Peptide-Specific CTLs. We investigated the ability of male NOD lymphocytes to serve as targets for K^d- or D^b-restricted CTLs specific for influenza virus. The susceptibility to CTL-mediated lysis of NOD male targets was similar to that of haplotype-matched control targets (Table 3). Class I molecules on male NOD lymphocytes therefore appear to present the correct peptide epitope for CTL lysis with an efficiency

Table 2. Susceptibility of female NOD and control strain target cells to class I-restricted influenza virus A/JAP-specific CTLs

Target cells	H-2	E:T	Specific lysis, % Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
			H-2D^b-restricted bulk CTLs (C57BL/6)				
NOD	D^b	10:1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	12 ± 3
C57BL/6	D^b	10:1	9 ± 1	9 ± 2	33 ± 5	28 ± 3	40 ± 6
B10.D2 (R103)	D^b	10:1	12 ± 2	30 ± 5	60 ± 8	63 ± 8	38 ± 3
			H-2K^d-restricted bulk CTLs (BALB/c)				
NOD	K^d	10:1	0 ± 0	0 ± 0	0 ± 0	32 ± 6	0 ± 0
B10.D2 (R103)	K^d	10:1	26 ± 5	—	50 ± 5	—	48 ± 4
BALB/c	K^d	10:1	9 ± 8	24 ± 3	48 ± 5	72 ± 10	—
			H-2K^d-restricted CTL clone 14-1				
NOD	K^d	5:1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	
B10.D2 (R103)	K^d	5:1	25 ± 1	33 ± 3	50 ± 5	33 ± 3	
BALB/c	K^d	5:1	12 ± 2	9 ± 0.5	14 ± 1	9 ± 3	

E:T, effector-to-target ratio.

comparable to control targets—as would be expected if Tap-1 functioned normally in these non-diabetes-prone mice. Two rare female NOD mice that consistently showed normal surface expression of class I molecules and, at the time of killing for these experiments, had normal Tap-1 and Tap-2 mRNA concentrations were also examined; one mouse successfully supplied peptide-loaded targets for D^b-restricted CTLs and the other mouse successfully provided targets for K^d-restricted CTLs.

Near-Normal Levels of Tap-1 mRNA in Male NOD Mice. Finally, we investigated the levels of Tap-1 mRNA in non-diabetes-prone male NOD who have normal expression of conformationally correct surface MHC class I on their lymphocyte surfaces compared to their female counterparts. As expected and in contrast to female NOD mice, male NOD mice have near-normal levels of Tap-1 mRNA (Fig. 4). A photomicrograph of Tap-1 mRNA expression in a single NOD female and male mouse compared to a control mouse demonstrated confinement of the depressed Tap-1 mRNA to only the female NOD mouse. This result was consistently observed in numerous Tap-1 mRNA quantitation experiments. As reported previously, Tap-2 mRNA levels are normal in the NOD mouse compared to control strains.

DISCUSSION

In summary, diabetes-prone female NOD mice exhibit reduced cell surface expression of conformationally correct class I molecules. Surface class I expression could be stabilized by low temperatures or by the addition of allele-specific peptides in female NOD mice, suggesting that the decreased density of conformationally correct class I on the surface of NOD lymphocytes is due to the lack of endogenous peptides in the class I groove for stabilization. Lymphocytes from diabetes-prone female NOD mice also showed a reduced rate of transport from the endoplasmic reticulum to the Golgi of class I molecules and failed to present internally derived cytoplasmic antigen fragments to CTLs. Target sensitivity to CTL lysis could be restored in female NOD cells by incubation with exogenous allele-specific peptide for the class I groove. These data are all consistent with a dysfunction of the peptide-empty class I molecule, and this perhaps could be due to direct or indirect dysfunction of the rare Tap-1 allele in female diabetes-prone NOD mice. In contrast, non-diabetes-prone NOD male mice showed normal class I expression at the cell surface, near-normal rates of egress of class I molecules from the endoplasmic reticulum, near-normal levels of Tap-1 mRNA, and normal susceptibility to class I-restricted peptide-specific CTLs. The simplest interpretation of these data is that the decreased expression of Tap-1 in female NOD mice and/or a mutant allele regulation hampers presentation of the correct number of self-peptides; this presentation of self-peptides in the groove of class I may be important for the induction of tolerance in the T-cell compartment and can be modified in part by sex influences. Since most autoimmune diseases demonstrate a marked predilection for females, these findings may be helpful in explaining variable penetrance between sexes. These presented functional studies in total suggest ATPase transporter malfunction in the diabetes-prone NOD mouse.

The mechanism underlying how dysfunction of an ATPase transporter causes diabetes or other autoimmune disease is speculative, since it now appears that the major class II-linked human autoimmune diseases all possess this functional interruption of class I self-peptide presentation (27). We suggest that interruption of cytoplasmically derived endogenous antigens either in a qualitative or quantitative manner could affect the T-cell repertoire and the education of T cells to self-antigens. Recent immunologic literature confirms this critical function of class I self-antigen presentation for CD8 cells as well as perhaps for CD4 cellular education (28, 29). Initial genetic studies based on Tap dysfunction in the human provide speculative but hopeful data that an important mutant in this region is possible (14, 30), and this could help to

Table 3. Effect of exogenous class I-specific peptide on susceptibility of NOD target cells to CTL lysis

Target strain	Sex	Exogenous peptide	Live virus	E:T	Specific lysis, % Exp. 1	Exp. 2	Exp. 3	Exp. 4
NOD	F	−	+	10:1	8 ± 6	0 ± 0	0 ± 0	6 ± 1
NOD	F	+	−	10:1	24 ± 2	9 ± 3	48 ± 5	33 ± 3
NOD	M	−	+	10:1	—	—	60 ± 5	20 ± 1
NOD	M	+	−	10:1	—	—	21 ± 2	100 ± 20
BALB/c	F	−	+	10:1	45 ± 7	15 ± 5	58 ± 20	20 ± 10
BALB/c	F	+	−	10:1	24 ± 4	39 ± 2	50 ± 10	100 ± 22

E:T, effector:target ratio.

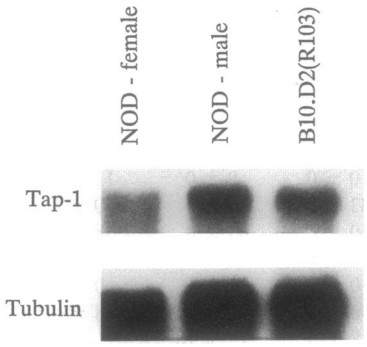

FIG. 4. Photomicrograph of a Northern blot of Tap-1 mRNA expression in a NOD female mouse and a NOD male mouse compared to a B10.D2 (R103) control mouse.

explain the decrease in conformationally correct class I molecules on the surface of diabetic human cells (23). The apparent presence of this same class I phenotypic defect in systemic lupus erythematosus, Graves disease, Hashimoto disease, Sjogren disease, and rheumatoid arthritis suggests a common and important interrupted pathway in self-antigen presentation (27).

In summary, we have demonstrated functional interruption of correct class I assembly and presentation of self-peptide in class I in the diabetes-prone NOD mouse, which is apparently quantitative. It remains to be demonstrated if this dysfunction is solely due to the *Tap-I* allele of the NOD female mouse and/or yet undetermined factors abnormally regulating these important genes or other genes involved in class I antigen processing and presentation.

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