# Expression of Intra-MHC Transporter (*Ham*) Genes and Class I Antigens in Diabetes-Susceptible NOD Mice

Although T lymphocytes are the ultimate effectors of pancreatic β cell destruction in the nonobese diabetic (NOD) mouse, immunoregulatory defects in antigen presentation probably provide the key to understanding the etiopathogenesis of autoimmune diabetes in this mouse model. *Ham*-1 and *Ham*-2 (also called *Tap-1* and *Tap-2*) (*1*) gene loci in the major histocompatibility complex (MHC) class II region, encode proteins required for normal antigen processing.

Faustman *et al.* report (*2*) genetic and functional defects in the *Ham*-1 gene of the NOD mouse and suggest that these defects constitute the MHC class II contribution to insulin-dependent diabetes mellitus (IDDM) susceptibility.

We have compared the *Ham*-1 and *Ham*-2 loci of the NOD/Lt mouse strain with those of other standard and wild-derived inbred strains (which represent a diverse collection of *H*-2 haplotypes) in order to establish the extent of the unique polymorphisms. To determine whether or not these polymorphisms are of pathogenic significance, we compared constitutive and cytokine-regulated *Ham*-1 gene transcription and translation in cultures of peritoneal macrophages and pancreatic β cell lines derived from NOD/Lt with those from control inbred mouse strains. Because one of the proposed (*3*) functions of HAM proteins is the transfer of antigenic peptides across the endoplasmic reticulum (ER) membrane in an adenosine triphosphate (ATP)–dependent fashion, we have also compared the ATP affinity of HAM molecules encoded by the NOD/Lt genome with that of an appropriate control.

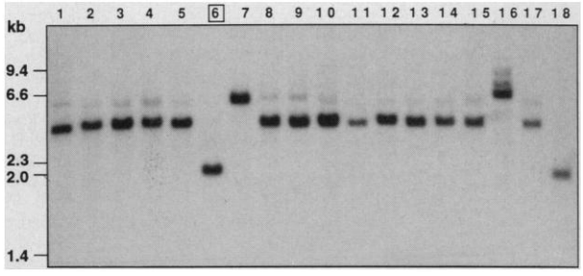


**Fig. 1.** Comparative RFLV analysis of *Ham*-1 gene structure among inbred mouse strains that represent different *H*-2 haplotypes. A Southern blot of genomic DNA from the indicated strains digested with Msp I was hybridized with a $^{32}$P-labeled genomic *Ham*-1 probe (5.9J) (*4*). The size (kilobases) of Hind III–digested λ phage DNA fragments is indicated on the left. Lane 1, A/HeJ; lane 2, C57BL/6J; lane 3, DBA/2J; lane 4, NZB/BINJ; lane 5, A.Ca/Sn; lane 6, NOD/Lt; lane 7, I/LnJ; lane 8, C3H/HeJ; lane 9, NON/Lt; lane 10, P/J; lane 11, SWR/J; lane 12, RIIIS/J; lane 13, PL/J; lane 14, SM/J; lane 15, NZW/LacJ; lane 16, *M. spretus*; lane 17, *M. poschiavinus*; and lane 18, CAST/Ei.

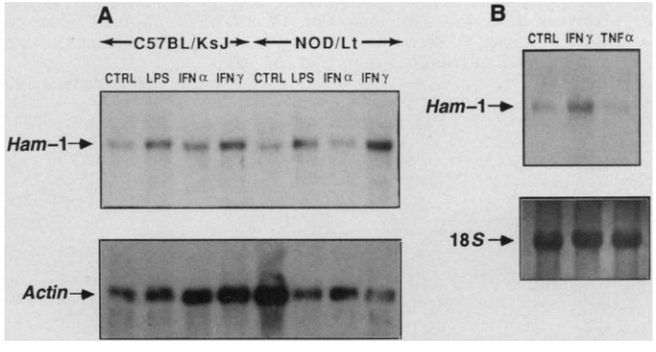


**Fig. 2.** Cytokine regulation of *Ham*-1 RNA expression in peritoneal macrophage cultures. (**A**) Thioglycollate recruited peritoneal macrophages from C57BL/KsJ and NOD/Lt mice were seeded at 5 × 10$^6$ cells per well in 24-well cluster plates and maintained in RPMI 1640 (Gibco BRL, Grand Island, New York) that contained 25 mM Hepes, 2 mM glutamine, 5 × 10$^{-5}$ M 2-mercaptoethanol (2-ME), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, Utah). Cultures were treated for 24 hours with medium alone as a control (CTRL), LPS (10 μg/ml), IFN-α (500 U/ml), or IFN-γ (50 U/ml). Total RNA preparations (10 μg per lane) were analyzed by sequential hybridization of a Northern blot with a $^{32}$P-labeled *Ham*-1 genomic probe (5.9B) (*4*) and an actin cDNA probe (*15*). (**B**) A Northern blot of total RNA from NOD/Lt peritoneal macrophage cultures treated for 24 hours with medium alone (CTRL) as a control, IFN-γ (50 U/ml), or TNF-α (100 U/ml) and then hybridized with the 5.9B *Ham*-1 probe. An ultraviolet light shadow, which illuminates 18*S* ribosomal RNA, shows equivalent loading of RNA in each lane. Rat recombinant IFN-γ was a gift from P. van de Meide (T.N.O. Primate Center, Rijswijk, The Netherlands). Mouse recombinant IFN-α and TNF-α were gifts from Hoffmann-La Roche (Nutley, New Jersey).

To identify distinguishing restriction fragment length variants (RFLVs), we compared DNA from NOD/Lt (cut with 27 restriction endonucleases) with DNA from NON/Lt, a related inbred strain that is resistant to IDDM. Southern blots were screened with genomic *Ham*-1 and *Ham*-2 probes (*3*). For *Ham*-1, digestion with Bst EII, Hpa I, Msp I, and Xba I yielded RFLVs between NOD and NON. For *Ham*-2, RFLVs between NOD and NON were detected with Bam HI, Stu I, and Xho I. To determine the uniqueness of these RFLVs, we typed Southern blots of DNA from 21 inbred and wild-derived mouse strains representing many of the known *H*-2 haplotypes with several of the distinguishing restriction enzymes (Fig. 1). The *Ham*-1 gene spans approximately 11 kb in genomic DNA (*4*). Of the enzymes that generate NOD RFLVs for *Ham*-1, only Msp I restriction sites have been found in the coding region for this gene. There are nine Msp I restriction sites present, including a site in one of two regions that encodes putative ATP-binding domains (*3*).

Combining the RFLV patterns obtained by the distinguished enzymes allows one to assign *Ham*-1 and *Ham*-2 alleles. On the basis of the restriction fragment sizes obtained after DNA digestion with Msp I and Xba I, four *Ham*-1 alleles (*Ham*-1$^a$ → *Ham*-1$^d$) were detected in the 21 mouse strains tested. The common allele was designated *Ham*-1$^a$, and the rare NOD allele was designated *Ham*-1$^b$. When Msp I and Xba I RFLV patterns were considered together, the *Ham*-1$^d$ allele of the I/LnJ strain appeared most unique among the inbred mouse strains tested. Of the four strains that exhibited a Msp I RFLV (Fig. 1), the NOD RFLV pattern was present only in the wild-derived inbred *Mus musculus castaneus* strain, CAST/Ei.

The *Ham*-2 gene was more polymorphic than the *Ham*-1 gene (Table 1). *Ham*-2 maps approximately 15 kb telomeric to *Ham*-1 in closer linkage to the polymorphic I-A region, which might explain the more polymorphic nature of this gene. On the basis of the restriction fragment sizes obtained after DNA digestion with Bam HI, Stu I, and Xho I, seven *Ham*-2 alleles were assigned (*Ham*-2$^a$ → *Ham*-2$^g$). The RIIIS/J strain was the only other inbred strain found to share the NOD allele, designated *Ham*-2$^d$. When individual enzyme RFLV patterns for *Ham*-2 were considered, a Xho I RFLV pattern for the Sm/J strain and a 10- kb Bam HI fragment for the POSCH/Ei strain were the only polymorphisms not shared by at least two mouse strains.

We performed Northern blot analysis to assess whether the rare NOD *Ham*-1$^b$ allele mediated reduced constitutive mRNA expression or defective responsiveness (or both) to induction by cytokines [interferon-α (IFN-α), IFN-γ, and tumor necrosis factor–α (TNF-α)] or by lipopolysaccharide (LPS) in cultured peritoneal macrophage cells (Fig. 2). RNA from C57BL/KsJ macrophages [which carry the *Ham*-1$^a$ allele common to most inbred strains (Table 1)] was used for comparison. Comparison of mRNA levels for both genotypes across all treatments shows that transcriptional function of the rare NOD *Ham*-1$^b$ allele was not impaired; the amounts of constitutive and inducible *Ham*-1 transcripts were comparable between the two strains (Figs. 2A and B). *Ham*-1 RNA expression was enhanced severalfold in macrophages from both strains after they were incubated for 24 hours with IFN-γ or LPS (Fig. 2A). In contrast to IFN-γ and LPS, *Ham*-1 RNA expression was not altered in macrophage cultures from either strain after they were treated for 24 hours with IFN-α (Fig. 2A) or in NOD macrophage cultures treated with TNF-α (Fig. 2B). When the same blots were probed with the *Ham*-2–specific probe, normal constitutive expression and cytokine upregulation of the rare *Ham*-2$^d$ allele in NOD macrophage cultures was also observed.

To assess the possibility that depressed *Ham* gene function could be cell-type specific, we also analyzed permanent pancreatic β cell lines established from transgenic mice (*5*). In contrast to the constitutive presence of *Ham*-1 and *Ham*-2 mRNA in macrophage cultures, neither *Ham* gene was expressed constitutively in pancreatic β cell lines. This lack of constitutive expression, which could distinguish the transformed β cell lines from primary macrophage cultures, was not a function of the transformed phenotype because no constitutive expression could be detected in primary islet cultures established from C3H/HeJ or from young NOD/Lt mice. As was observed for macrophage cultures, *Ham* gene transcription was strongly induced by IFN-γ in primary islet cultures. In pancreatic β cell lines, a maximum *Ham*-1 inductive response was observed after only a 2-hour exposure to IFN-γ (Fig. 3). For all of the time periods examined, *Ham*-1 RNA transcripts were detected only in cultures treated with IFN-γ. IFN-γ–induced *Ham*-1 RNA expression varied over 6 days of cytokine treatment. The rapid induction of *Ham*-1 expression in IFN-γ–treated β cells, which normally express low amounts of MHC class I, supports the hypothesis that *Ham*-1 gene products are necessary for events early in MHC class I maturation. In contrast to the early *Ham*-1 response, inductive effects for MHC class I (*H-2 K/D*) and $\beta_2$-microglobulin ($\beta_2$M) were observed after exposure for 24 hours to IFN-γ (Fig. 3). For both MHC class I and $\beta_2$M, RNA transcript accumulation peaked after 3 days of IFN-γ treatment. MHC class II and I invariant chain RNA inductive patterns, formed in response to IFN-γ, mimicked those observed for MHC class I and $\beta_2$M genes. *Ham*-1 RNA induction was similar in the βTC1 cell line. We chose it as a control because it expresses the MHC genes from both the C57BL/6J and DBA/2J parental strains (both H-2 haplotypes carry the common *Ham*-1$^a$ allele). Moreover,

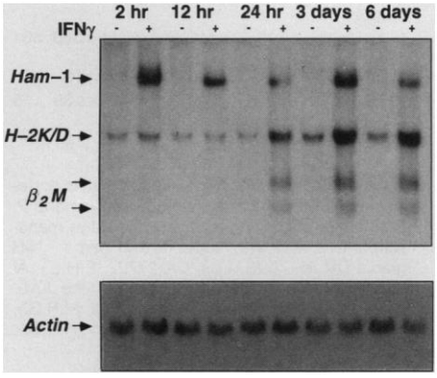


**Fig. 3.** Time course of IFN-γ–induced RNA expression of *Ham*-1, MHC class I (*H-2K/D*), $\beta_2M$, and *actin* in the NOD-derived pancreatic β cell line, NIT-1. NIT-1 cultures were incubated for the indicated times in the presence (+) or absence (−) of IFN-γ (50 U/ml). Total RNA preparations (10 μg/lane) were analyzed by sequential hybridization of a Northern blot with $^{32}$P-labeled genomic or complementary DNA probes that recognize these four transcripts (*16*).

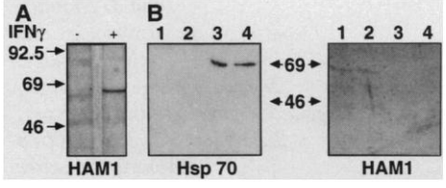


**Fig. 4.** The HAM1 protein is induced by IFN-γ in pancreatic β cell lines, but does not bind ATP in in vitro binding assays. (**A**) The effect of IFN-γ on HAM1 expression in βTC3 cells was determined by protein immunoblot analysis with a rabbit antiserum to a synthetic HAM1 oligopeptide (peptide B, amino acids 215 to 225) (*3*). βTC3 cultures were treated for 2 days with (+) or without (−) IFN-γ (50 U/ml). Cells were harvested and sonicated in 0.1% Triton-X 100 in phosphate-buffered saline, and proteins were size-separated by one-dimensional 12.5% SDS-PAGE. Gels were blotted onto Immobilon-P membranes (Millipore, Bedford, Massachusetts). A mouse monoclonal antibody (MAb) (N27F3.4) that recognizes both the constitutive (73 kD) and inducible (72 kD) forms of hsp70 was provided by W. Welch. Primary Abs were N27F3.4 (1/5000) and HAM1 anti-peptide B, (1/500). Biotinylated secondary Abs (1/2000) to rabbit Immunoglobulin G (IgG) and to mouse IgG (Vector Laboratories, Burlingame, California) detected Abs to peptide B and MAb N27F3.4, respectively. The alkaline phosphatase-avidin D (0.2 U/ml; Vector) was developed with bromochloroindolyl phosphate/nitro blue tetrazolium chromogenic substrate (Boehringer Mannheim, Indianapolis, Indiana). (**B**) Cell lysates from βTC3 and NIT-1 cultures were treated for 4 days with IFN-γ (50 U/ml), incubated with ATP-agarose at 4°C for 4 hours, and eluted into fractions that represented degrees of ATP affinity. Duplicate immunoblots were immunostained for hsp70 or HAM1 as described above. Lanes 1 (βTC3) and 2 (NIT-1) are the unbound fractions after incubation with ATP-agarose. Lanes 3 (βTC3) and 4 (NIT-1) are the fractions that remained bound to ATP-agarose after high salt washes. For both (A) and (B) the molecular sizes of the following protein standards are indicated: phosphorylase b, 92.5 kD; bovine serum albumin, 69 kD; and ovalbumin, 46 kD.

**Table 1.** Inbred strain distribution of *Ham*-1 and *Ham*-2 alleles. Ham-1 and *Ham*-2 alleles were assigned from the combined distribution of the following restriction endonuclease fragment sizes. The *Ham*-1 Msp I fragments identified with probe 5.9J were either 3.8 kb (a), 2.3 kb (b), or 4.5 kb (c and d), and identified with probe 5.9B were 1.0 and 1.6 kb (a to d). The *Ham*-1 Xba I fragments identified with probe 5.9J were either 10.0 kb (a) or 8.0 kb (b, c, and d), and identified with probe 5.9B were either 10.0 kb (a) or 8.0 and 4.4 kb (b, c, and d). The *Ham*-2 Bam HI fragments were either 4.3 kb (a to c, e, and f), 3.4 kb (d), or 10.0 kb (g). The *Ham*-2 Stu I fragments were either 9.5 and 8.8 kb (a, b, and f), 9.5 and 4.0 kb (c, d, and g), or 9.5 kb (e). The *Ham*-2 Xho I fragments were either 15.0, 9.5, and 5.5 kb (a), 17.0, 9.7, and 8.0 kb (b to e, and g), or 22.0, 17.0, 12.0, and 9.7 kb (f).

| Strain | *H-2* haplotype | *Ham*-1 allele | *Ham*-2 allele |
|---|---|---|---|
| A/HeJ | a | a | a |
| C57BL/6J | b | a | c |
| C57BL/KsJ | d | a | nd |
| BALB/cByJ | d | a | nd |
| DBA/2J | d | a | e |
| NZB/BINJ | d | a | e |
| A.CA/Sn | f | a | b |
| NOD/Lt | g[7] | b | d |
| I/LnJ | j | d | c |
| C3H/HeJ | k | a | a |
| NON/Lt | nd[1] | a | e |
| P/J | p | a | a |
| SWR/J | q | a | c |
| RIIIS/J | r | a | d |
| PL/J | u | a | c |
| SM/J | v | a | f |
| NZW/LacJ | z | a | c |
| *M. spretus* | nd | c | e |
| POSCH/Ei | nd | a | g |
| CAST/Ei | nd | b | b |
| MOLF/Ei | nd | a | a |

*M. spretus* DNA samples were from a partially inbred colony maintained by J. Nadeau at The Jackson Laboratory, Bar Harbor, Maine. Not determined, nd.

when Northern blots containing RNA from NIT-1 and βTC1 cells were hybridized with the *Ham*-2–specific probe, equivalent induction of *Ham*-2 transcripts was observed. Thus, the rare NOD *Ham*-$1^b$ and *Ham*-$2^d$ alleles carried in the NIT-1 cell line did not prohibit transcriptional induction by IFN-γ. HAM1 protein expression was also induced in β cell lines in response to IFN-γ (Fig. 4A). A single band of approximately 65 kD corresponding to HAM1 was detected only in cell lysates from IFN-γ–treated cultures. A comparable IFN-γ inductive effect on HAM1 protein was observed in βTC3 and NIT-1 cell cultures (Fig. 4B).

Two putative ATP-binding cassettes are present in the HAM1 protein sequence (*3*); however, HAM1 affinity for ATP has not been reported. We used an ATP-agarose purification technique (*6*) to compare the ATP affinity of HAM1 to that of a known high affinity ATP-binding protein, heat shock protein 70 (hsp70). As expected, the constitutive hsp70 protein remained bound to the ATP-agarose even after extensive washing with high salt buffer (2 M NaCl), which demonstrated the high ATP affinity of this molecule (Fig. 4B). In contrast, the HAM1 protein was detected only in the unbound lysate, which indicated low or no affinity for ATP under the assay conditions (Fig. 4B).

These results contrast with Faustman *et al.*'s conclusion that the Xba I RFLV associated with the NOD *Ham*-$1^b$ allele represented a mutation that impaired gene function (*2*). Faustman *et al.* further propose that reduced *Ham* RNA expression is contributing to MHC-linked autoimmune diabetes susceptibility on the basis of their observation of reduced cell surface expression of MHC class I molecules on NOD splenocytes. However, our results show normal constitutive and cytokine regulated expression of *Ham*-1 in NOD/Lt peritoneal macrophage cultures. In addition, although cultured β cells (including the NIT-1 cell line and primary islet cultures) differed from macrophages in that they did not exhibit constitutive *Ham*-1 expression, we found that this expression was IFN-γ–inducible and that the response was not impaired in NIT-1 cells. Finally, the fact that IFN-γ treatment of a NOD/Lt β cell line produced HAM1 molecules of correct molecular size confirmed that *Ham*-1 mRNA was translated. Consistent with normal regulation of *Ham* loci, we observed nominal levels of MHC class I antigen on the cell surfaces of freshly isolated splenic leukocytes from prediabetic NOD/Lt mice (*7*). It is generally accepted that RFLVs only denote differences in restriction enzyme sites in homologous DNA regions. Thus, in the absence of precise comparison of gene sequences to establish the relation of unusual *Ham*-1 restriction endonuclease sites to transcribed regions, altered fragments sizes should not necessarily be considered synonymous with function-abrogating mutations in coding or regulatory regions.

Both the *Ham*-1 and *Ham*-2 genes are required for antigen processing, as transfection of these genes into processing-defective cells restores antigen processing capability (*8*). Although our results, which we obtained with cells in vitro, demonstrate normal *Ham*-1 gene expression, HAM function in NOD mice could nonetheless be abnormal. For example, the *Ham* genes within the *H*-$2^{g7}$ haplotype could contribute to diabetogenesis by influencing the repertoire of peptides available for presentation by MHC class I molecules. Altered ATP-binding affinities for NOD *Ham* gene products may also exist. However, we were unable to demonstrate an ATP affinity for NOD or control strain–derived HAM1 proteins, so the lack of HAM1 binding may necessitate a reconsideration of the hypothesis that HAM molecules function as ATP-dependent peptide pumps (*3*), which is consistent with a recent report (*9*).

Extended haplotype analysis of *H*-$2^{g7}$ continues to reveal the presence of other unique alleles that may also contribute to the diabetogenic potency of this haplotype (*10–12*). In addition, many intra-MHC genes required for distinct aspects of antigen processing are now being revealed (*4, 13, 14*). We propose that detailed investigation of the structure and regulated expression of these genes in the diabetogenic *H*-$2^{g7}$ haplotype will finally link genetic regions and immunodysfunctions that characterize autoimmune diabetes in the NOD mouse.

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## REFERENCES AND NOTES

1. A recent HLA Nomenclature Committee meeting resolved that the names *Tap-1* and *Tap-2* (for *t*ransporter associated with *a*ntigen *p*rocessing) be used for *Ham*-1 and *Ham*-2 and for their homologs in rats and humans [J. J. Monaco, *Immunol. Today* **13**, 173 (1992)].
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Faustman *et al.* propose (*1*) that faulty expression of major histocompatibility complex (MHC) class I molecules is linked to autoimmune diabetes. They found (*1*) decreased class I expression in both 6-week-old normoglycemic and 27-week-old hyperglycemic nonobese diabetic (NOD) mice as compared with that in BALB/c mice or in 27-week-old normoglycemic NOD mice. Two of the monoclonal antibodies (MAb) used by Faustman *et al.* were SF1-1.1 (PharMingen, San Diego, California) and 9010A (Accurate Chemical, Westbury, New York), both of which react with the class I product $K^d$.

Our studies of the NOD mouse model of type 1 diabetes have used NOD/MrkTac mice, which develop a high incidence of diabetes when housed in our facility (>80% of females and 50% of males by 7 months of age) (*2*). The NOD mouse, which has the *H*-$2^{g7}$ MHC haplotype, expresses the cell surface antigens $K^d$, I-$A^{g7}$, and $D^b$. We have identified one of the class I products of the NOD mouse with SF1-1.1, a MAb of the IgG2a isotype reported to react with $K^d$ but not the H-$2^{b,f,j,k,p,q,r,s,u,\text{ or } v}$ haplotypes (*3*). We have also used MAb SF1-1.1 to develop two reciprocal MHC congenic strains of mice: B10.H-$2^{g7}$ (*2*) and NOD.H-$2^b$ (*4*). As expected, B10.H-$2^{g7}$ spleen cells, but not NOD.H-$2^b$ spleen cells, were

**Table 1.** MHC Class I expression on splenic T and B cell subpopulations. MFI, mean fluorescence intensity of MHC class I expression of the cells in the subpopulation. Spleen cells were incubated with phycoerythrin-labeled monoclonal antibodies, which recognize the T cell surface markers CD4 (clone RM-4-5, PharMingen) and CD8 (clone 53-6.7, PharMingen) or which recognize the B cell surface marker B220 (clone DNL-1.9, PharMingen). Spleen cells were incubated in a volume of 0.1 ml with 2 μg of antibody to CD4, 0.4 μg of antibody to CD8, antibody to CD4 and antibody to CD8 together, or 0.24 μg of antibody to B220. The cells were incubated with the phycoerythrin-labeled antibodies for 20 min at 4°C and then with fluorescein-labeled antibody to $K^d$, as described in Fig. 1. The mean fluorescence intensity of class I expression in each subpopulation was obtained by gating on phycoerythrin-positive cells.

| Mouse strain and age | Cell subpopulations represented in the spleen % positive (MFI) | | | |
|---|---|---|---|---|
| | CD4 | CD8 | CD4/CD8 | B220 |
| BALB/c 25 weeks | 19.5 (140) | 7.2 (126) | 27.1 (136) | 65.0 (177) |
| NOD 6 weeks | 35.3 (114) | 12.5 (103) | 45.6 (107) | 43.9 (165) |
| NOD 16 weeks | 32.2 (123) | 14.1 (102) | 45.6 (120) | 44.2 (227) |

recognized by the SF1-1.1 antibody (*4*).

Faustman *et al.* (*1*) found that MAb SF1-1.1 recognized spleen cells from 6-week-old NOD mice [(*1*), figure 1A] and C57BL/6 mice [(*1*), figure 1C] in an equivalent manner; 33% of C57BL/6 and 23% of 6-week-old NOD spleen cells reacted with MAb SF1-1.1. In addition, they found the mean channel fluorescence for the positive NOD cells to be 20% of that for the positive control BALB/c spleen cells [(*1*), figures 1A and E]. In contrast, we observed that MAb SF1-1.1 reacted with spleen cells from 6-week-old NOD mice but not with spleen cells from C57BL/6 mice. MAb SF1-1.1 bound to spleen cells from a BALB/c mouse (Fig. 1A), a 6-week-old NOD mouse (Fig. 1B), an 18-week-old normoglycemic NOD mouse (Fig. 1C), and a 22-week-old hyperglycemic mouse (Fig. 1D) with essentially equivalent mean fluorescence intensities of 281, 292, 274, and 270, respectively. We found a lack of reactivity by MAb SF1-1.1 on spleen cells from a C57BL/6 mouse (Fig. 1E). This last result is in contrast to the "expected cross-reactivity" described by Faustman *et al.* (*1*) on C57BL/6 spleen cells but in agreement with the specificity described for this MAb (*3*). Spleen cells obtained from the BALB/c mouse and the three NOD mice did not bind to monoclonal IgG2a antibody, AF6-88.5 [which reacts

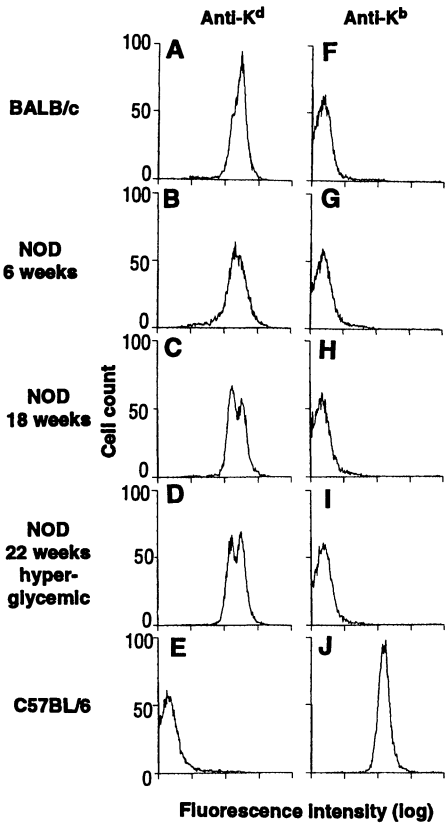


**Fig. 1.** Dissociated spleen cells ($10^6$), depleted of red blood cells, were incubated with 0.53 μg of fluorescein-labeled murine MAb SF1-1.1 (anti-$K^d$; Pharmingen) or 2 μg of fluorescein-labeled murine MAb AF6-88.5 (anti-$K^b$; PharMingen). Saturating amounts of both antibodies were used. After incubation with the antibodies for 20 min at 4°C, the cells were washed and analyzed by flow cytometry on the FACStar$^{PLUS}$ (Becton-Dickinson, Mountainview, California). Propidium iodide was added to eliminate dead cells. Mice were purchased from Taconic Farms (Germantown, New York) and housed under sterile, specific pathogen-free conditions. The BALB/c and C57BL/6 mice were 17 and 9 weeks of age, respectively. All mice were female except for the diabetic NOD mouse.

with $K^b$ but not with the H-$2^{d,f,j,k,p,q,r,s,u,\text{ or } v}$ haplotypes (PharMingen)], whereas spleen cells from the C57BL/6 mouse were positive (Fig. 1, F to J).

We therefore conclude that spleen cells obtained from 6-week-old NOD mice and from hyperglycemic NOD mice do not express reduced amounts of the $K^d$ class I antigen as compared with normoglycemic NOD mice or BALB/c mice. Thus, the hypothesis that faulty or reduced MHC class I expression on spleen cells is linked to autoimmune diabetes in the NOD mouse (*1*) is not supported by our results.

Although MAb SF1-1.1 did react with BALB/c spleen cells and with all sources of NOD spleen cells, saturating amounts of the antibody exhibited different patterns of reactivity on different cell populations (Fig.

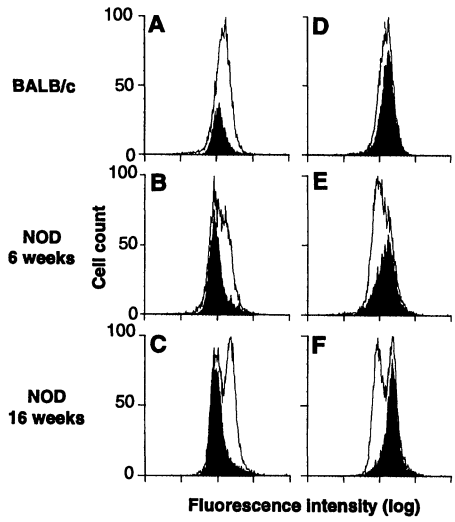


**Fig. 2.** Class I expression on splenic T and B cell subpopulations. Before incubation with fluorescein-labeled antibody to $K^d$, spleen cells ($10^6$) were incubated with phycoerythrin-labeled antibody to CD4 and antibody to CD8 (added together) to identify T cells or with phycoerythrin-labeled antibody to B220 to identify B cells (see Table 1 for antibody concentrations). In (**A**) to (**C**), class I expression on all spleen cells from the mice indicated is compared with that on $CD4^+/CD8^+$ cells within the spleen cell population (darkened profile in each panel). In (**D**) to (**F**) class I expression on all spleen cells is compared with class I expression on $B220^+$ cells (darkened profiles). The mean fluorescence intensity of class I expression on each cell subpopulation was obtained by gating on phycoerythrin-positive cells. The data in Table 1 and Fig. 2 are from the same experiment.

1). Therefore we examined class I expression on splenic T and B cell subpopulations by two-color fluorescence analysis (Table 1; Fig. 2). Both BALB/c and NOD $B220^+$ B cells expressed 30 to 90% higher amounts of class I antigen expression than either $CD4^+$ or $CD8^+$ T cells. Because NOD mice have a higher percentage of T cells in their spleen compared with BALB/c mice (45.6 as opposed to 27.1%) (Table 1), a higher proportion of NOD spleen cells would be expected to express lower amounts of class I antigen as compared with BALB/c spleen cells.

Class I expression on NOD T cells is slightly lower (10 to 20%) than on BALB/c T cells, whereas class I expression on B cells from 6-week-old NOD mice is nearly identical to that on BALB/c B cells (Table 1). By 16 weeks of age, however, the B cells from NOD mice exhibit 40% more class I expression than B cells from 6-week-old NOD mice (Table 2). This increase in B cell class I expression produces the prominent bimodal distribution observed in spleen cells obtained from NOD mice greater than 6 weeks of age (Figs. 1 and 2).

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3. The SF1-1.1 hybridoma cell line was submitted to the American Type Culture Collection (ATCC) by A. M. Stall, Stanford University. As noted in the ATCC catalog, SF1-1.1 secretes an IgG2a monoclonal antibody that reacts with H-2K$^d$.
4. L. S. Wicker *et al.*, *J. Exp. Med.*, in press.

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*Response*: To directly compare MHC class I expression in NOD and control BALB/c mouse splenocytes, we superimposed the histograms from figure 1 of Wicker *et al.* in our Fig. 1. The expression in mice of 6 weeks (Fig. 1A), 18 weeks (Fig. 1B), and 22 weeks (Fig. 1C) of age was clearly different in NOD mice from that in control mice of the same ages; the histograms for the three NOD mice age groups show a decreased peak height and a biphasic peak pattern. Therefore, the data of Wicker *et al.* seem to confirm our finding (*1*) of a difference in MHC class I expression in NOD splenocytes as compared with that in BALB/c splenocytes.

Wicker *et al.* performed MHC class I phenotyping on NOD splenocytes with a directly conjugated MHC class I MAb SF1-1.1 (PharMingen, San Diego, California), and demonstrated decreased background cross-reactivity with negative C57BL/6 splenocytes. Consistent with standard immunological procedures, their data show that the high background immunofluorescence of mouse splenocytes, observed with the use of FITC-conjugated goat antibodies to mouse immunoglobulin, can be eliminated with MAb SF1-1.1. When we used directly conjugated MAb SF1-1.1, we observed MHC class I data identical to that obtained by Wicker *et al.* As expected, we also observed decreased cross-reactivity with C57BL/6 splenocytes. Nevertheless, we observed a diminished MHC class I density with both techniques in the NOD mouse.

In humans with diabetes, lymphocytes show consistent defective expression of MHC class I antigens. However, in NOD mice splenocytes, different antibodies to MHC class I antigens show varying degrees of defective MHC class I expression. The 9008-A antibody to H-2K$^d$, which binds to the epitope binding region of the K$^d$ molecule, has shown a more striking defect in MHC class I expression in NOD splenocytes than has MAb SF1-1.1 (Fig. 2A).

We prospectively monitored 201 females in our colony of NOD mice with a high incidence of diabetes from 4 to 6 weeks of age with 9008-A antibody to test the predictive power of this change in MHC class I reactivity with regard to disease penetrance. We found by flow cytometric analysis of 169 mice that MHC class I density decreased on peripheral blood lymphocytes, as reported by Gaskins *et al.* (Fig. 2A); the remaining 32 mice showed MHC class I expression patterns identical to those of control BALB/c mice (Fig. 2B). By 9 months of age, 100% of the 169 mice that showed modified MHC class I expression developed severe hyperglycemia and died. At 18 months of age, the 32 mice with normal lymphocyte cell surface expression of MHC class I remained normoglycemic. These results are similar to our data for human identical twins discordant for type I diabetes (*1*). Therefore, diminished expression of MHC class I antigens observed on the surface of peripheral lymphocytes is predictive of the progression to hyperglycemia in genetically susceptible populations of both humans and mice.

Another important technical point is that exposure of murine splenocytes to serum-containing media at room temperature

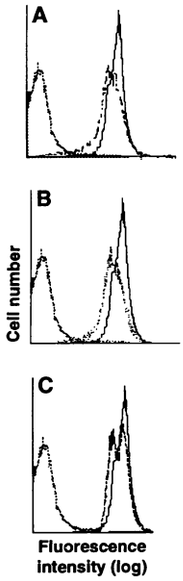


**Fig. 1.** MHC class I expression in NOD mice (dashed lines) and BALB/c mice (solid lines) (**A**) 6 weeks, (**B**) 18 weeks, and (**C**) 22 weeks of age. Data from figure 1 of Wicker *et al.* for NOD and BALB/c mice of various ages have been superimposed for comparison.

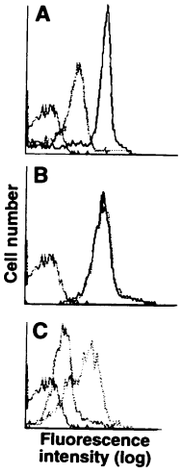


**Fig. 2.** Immunofluorescence on splenocytes from NOD mice and BALB/c mice obtained with fluorescein isothiocyanate–conjugated antibody to H-2K$^d$ (CL9008-A, Accurate Chemical, Westbury, New York). (**A**) NOD mouse splenocytes (dashed line) compared to BALB/c splenocytes (solid line) show decreased MHC class I expression. (**B**) Splenocytes from a female NOD mouse that did not develop hyperglycemia by 18 months of age (dashed line) compared to those from a BALB/c control (solid line). (**C**) Splenocytes from an NOD mouse with defective MHC class I expression before (left dashed line) and after (right dashed line) incubation for 4 to 5 hours in medium containing 10% fetal bovine serum at room temperature. Background immunofluorescence was less than 5% for all BALB/c and NOD splenocytes (far left peak in each histogram).

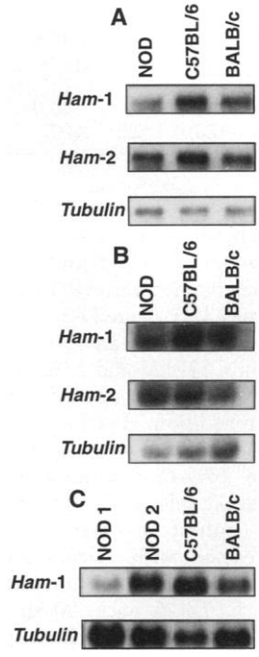


**Fig. 3.** Northern blot analysis of *Ham*-1 and *Ham*-2 mRNA concentrations in spleens of NOD, C57BL/6, and BALB/c mice. The mRNA was rapidly prepared from whole spleens after the mice were killed (**A**). The *Ham*-1 probe (a murine homolog of *Ring*-4) demonstrates the specifically suppressed mRNA levels in the NOD mouse (*1*). (**B**) *Ham*-1 and *Ham*-2 mRNA concentrations in NOD, C57BL/6, and BALB/c mouse spleen cells exposed to media containing serum for 4 to 5 hours at room temperature before mRNA preparation. (**C**) *Ham*-1 mRNA concentration in splenocytes from an NOD mouse with defective MHC class I expression (NOD 1) compared with that of splenocytes from an NOD mouse with normal MHC class I expression (NOD 2).

**Table 1.** MHC class I expression on splenic lymphocyte and macrophage cell subpopulations. MAD, mean antigen density (or mean channel fluorescence), is the density of MHC class I determinants. NOD, BALB/c, and C57BL/6 female mice were 8 to 12 weeks old. MAb to H-2K$^d$ was purified and directly conjugated to fluorescein isothiocyanate (FITC) (CL9008-A, Accurate Chemical). It recognizes cells bearing the H-2K$^d$ (H-2.31) antigen (Accurate Chemical). MAb H-2D$^b$ was also purified and directly conjugated to FITC (CL9002-A, Accurate Chemical). It recognizes H-2.m2b(3). Macrophages were identified with MAb M1170.15, which reacts with polypeptides of 190 and 105 kD, which are found exclusively on macrophages. MAb M1170.15 was conjugated to phycoerythrin for two-color immunofluorescence (Coulter, Hialeah, Florida). Mouse lymphocytes were identified as cells expressing CD4-RD1 or CD8-RD1 (PharMingen). Background immunofluorescence was less than 5% for all antibodies on lymphocytes from different mice.

| Mouse strains | Cell subpopulations represented in the spleen % positive, (MAD) | | Mouse strains | | |
|---|---|---|---|---|---|
| | H-2K$^d$(CL9008-A) | | | H-2D$^b$ (CL9002-A) | |
| | Lymphocytes | Macrophages | | Lymphocytes | Macrophages |
| BALB/c | 99 (2.7) | 98 (31) | C57BL/6 | 99.7 (19) | 99.5 (52) |
| NOD | 44 (1.6) | 93 (13.5) | NOD | 52 (13) | 92.5 (41) |
| BALB/c | 97 (5.7) | 98 (40) | C57BL/6 | 99.8 (18.7) | 100 (50) |
| NOD | 46 (3.1) | 94 (21) | NOD | 62 (15) | 91.5 (34) |
| BALB/c | 98 (6.9) | 100 (50) | C57BL/6 | 99.6 (18.5) | 100 (49) |
| NOD | 66 (3.0) | 99 (34) | NOD | 59 (16) | 98 (32) |

for 3 to 5 hours before flow cytometer analysis partially corrects the defective pattern of MHC class I expression (Fig. 2C). This finding is consistent with the recent work of Ljunggren *et al.*, which shows that the labile peptide-empty MHC class I on mutant murine lymphoma cells can be stabilized by reduced temperatures (*2*). Although diminished MHC class I expression in NOD mice is evident with all MHC class I antibodies tested, the apparent magnitude of the decrease varies depending on the specific MHC class I antibody used. Defective MHC class I expression is observed whether measured indirectly, with a second antibody, or with a direct assay with a fluorescent-conjugated primary antibody. Incubation of the NOD lymphocytes in serum for 3 to 5 hours at room temperature diminishes the magnitude of the defect in MHC class I expression.

With MAb SF1-1.1, Wicker *et al.* found MHC class I expression to be decreased in T cells but normal in B cells. However, MAb SF1-1.1 appears to be an exception; the majority of antibodies to H-2K$^d$ or H-2D$^b$ reveal decreased MHC class I expression on NOD antigen–presenting cells. Antibody 9008-A to H-2K$^d$ and antibody 9002-A to H-2D$^b$ reveal decreased MHC class I expression in T cells and in antigen-presenting cells from NOD mice (Table 1). These data then confirm our earlier conclusion: The lymphocytes of the NOD mouse have a defect in MHC class I expression.

We have also reported data (*1*) which suggest that this defective MHC class I expression may be controlled by a series of MHC class II–linked transporter genes. The transporters encoded by these genes regulate the delivery of cytoplasmically processed peptides into the endoplasmic reticulum for combination with MHC class I molecules. To generate these data, we rapidly prepared mRNA from NOD splenocytes and used the human *RING*-4 transporter probe to measure mRNA for murine MHC class II–linked transporter genes. In all instances, mRNA prepared from NOD mice whose lymphocytes showed decreased MHC class I expression also showed decreased transporter mRNA concentrations (*1*).

With the availability of murine probes to the two MHC class II–linked transporter genes *Ham*-1 (the murine homologous of *RING*-4) and *Ham*-2, we have found that the low transporter mRNA concentrations in NOD splenocytes reflect decreased expression of *Ham*-1, while *Ham*-2 mRNA concentrations are normal (Fig. 3A). Furthermore, *Ham*-1 mRNA concentrations can be rapidly restored to near-normal values by exposing splenocytes to 10% serum at room temperature for 4 to 5 hours before mRNA preparation (Fig. 3B).

Because the defective MHC class I phenotype is predictive of progression to hyperglycemia, it might therefore be expected that NOD mice with normal MHC class I surface expression that do not develop diabetes would also show normal expression of *Ham*-1 mRNA. Indeed, we have found that such mice have normal *Ham*-1 mRNA concentrations and NOD mice with decreased MHC class I expression have low *Ham*-1 mRNA concentrations.

Gaskins *et al.* conclude that macrophages from NOD mice have normal *Ham*-1 mRNA concentrations. However, with their methods, one would expect macrophages isolated from immunologically stimulated NOD mice and cultured in the presence of serum to show normal *Ham*-1 mRNA. A perplexing variety of immune stimulation protocols prevents diabetes in the NOD mouse (*3*–*6*). Future studies are sure to be directed at restoration of MHC class I expression or *Ham*-1 induction at a mechanism for successful treatment.

Gaskins *et al.* appear to confirm our NOD Southern analysis data and to demonstrate the previously described (*1*) Xba I DNA site of the *Ham*-1 (*Ring*-4) transporter locus. In addition, they expand our data and demonstrate that this polymorphism is rare but not unique and that this mutation does not affect the coding region.

Therefore, the NOD mouse, like humans with type I diabetes, shows defective MHC class I expression in lymphocytes, including antigen-presenting cells in vivo. This phenotype is correlated with low concentrations in vivo of *Ham*-1 mRNA and diabetes progression. We have hypothesized (*1*) that MHC class II–linked transporter loci control the delivery of endogenous peptides into the endoplasmic reticulum, which presumably allows correct folding of MHC class I molecules and sub-sequent expression on the cell surface. Interruption of this pathway appears to be associated with disease penetrance.

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7. We thank J. J. Monaco for the *Ham*-1 and *Ham*-2 probes and L. Murphy for secretarial assistance.

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