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Crosses of two independently derived transgenic mice demonstrate functional complementation of the genes encoding heavy (HLA-B27) and light (β_2-microglobulin) chains of HLA Class I antigens

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In man a number of diseases are associated with certain alleles of MHC antigens. The most pronounced example is ankylosing spondylitis, which is strongly associated with HLA-B27. As a first step towards a model system to study the basis of this association, transgenic mice were generated that showed cell surface expression of the HLA-B27 antigen biochemically indistinguishable from HLA-B27 antigen expressed on human cells. This result was obtained by crossing two independently derived strains of mice, one of which is transgenic for the HLA-B27 heavy chain gene, and the other carrying and expressing the human β_2m gene. Examination of HLA-B27 and human β_2m mRNA in various tissues shows the two genes to be expressed in a coordinate fashion. The mRNA levels follow those of endogenous H-2 Class I genes.

Key words: histocompatibility antigens/transgenic mice/human β_2m/HLA-B27

Introduction

Histocompatibility antigens play a pivotal role in establishing productive interactions between T lymphocytes and their targets. The polymorphism of histocompatibility antigens is thought to be linked functionally to the individual's ability to mount an immune response against certain antigens. Indeed, certain strains of mice are unable to ward off infections by Sendai virus, depending on their major histocompatibility complex (MHC) haplotype (Kast *et al.*, 1986). Similarly, in man a number of diseases with an autoimmune component to their etiology are associated with particular alleles of MHC antigens (Svejgaard *et al.*, 1983). The best known example is ankylosing spondylitis, which is associated with HLA-B27 (Brewerton *et al.*, 1973), yet nothing is known about the mechanistic basis of this association. Infection with gram-negative bacteria such as *Klebsiella spp.* have been implicated, but no suitable model system amenable to the study of causal relationships exists (Moeller, 1985). Mice transgenic for HLA-B27 might contribute towards establishing such model systems.

In studies on the expression in mouse cells of cloned HLA-B27 genes, it was established that the presence of both human subunits [heavy and light (β_2-microglobulin) chains] was required to achieve the formation of a complex which was expressed efficiently at the cell surface. This is unlike other human Class I MHC genes (Rein *et al.*, 1987). Any strategy aimed at expressing histocompatibility antigens in heterologous systems has to take into account this possibility of hybrid antigens, and thus antigenically and perhaps functionally distinct, molecules. Human and mouse β_2m are only 70% homologous (Ploegh *et al.*, 1981).

Mice expressing HLA-B27 antigens as the offspring of two strains, one of which is transgenic for the HLA-B27 heavy chain, and the other carrying and expressing the gene encoding the human light (β_2m) chain were produced. These results also provide genetic proof of the requirement for subunit association in the expression of Class I antigens at the cell surface.

Results and Discussion

Expression of genes encoding HLA-B27 heavy and light chains in transgenic mice

It has been demonstrated that the introduction of the HLA-B27 gene alone into mouse 3T3 or Ltk− cells does not result in high levels of expression of correctly assembled and processed Class I molecules (Rein *et al.*, 1987; Seemann *et al.*, 1986). But the subsequent introduction of a cloned human β_2m gene produced high levels of HLA-B27 antigen indistinguishable from that derived from human cells (Rein *et al.*, 1987). We therefore decided to construct two lines of transgenic mice, one carrying the HLA-B27 gene and the other the human β_2m gene, using the pronuclear injection technique. A physical map of both gene constructs used for injection is given in Figure 1. Southern blot analysis of chromosomal tail DNA from mice that developed from microinjected eggs identified 12 animals carrying the HLA-B27 gene and 12 transgenics for the human β_2m gene. The results of this analysis for the HLA-B27 and the human β_2m transgenic mice that were used for further breeding, are depicted in Figure 1.

Transgenic mice were splenectomized and splenocytes were isolated for further analysis. By examination of expression at the RNA and protein level all HLA-B27 transgenic animals were positive on all counts. In contrast, only two animals harboring the human β_2m gene produced specific mRNA and protein (data not shown). We have no satisfactory explanation for the difference in efficiency in obtaining expression of the transgenes, although the failure to remove plasmid sequences prior to injection of the human β_2m gene may contribute (Shani, 1986).

Animals that were positive for expression of mRNA and protein (mouse 22 carrying the HLA-B27 gene and mouse 55 carrying the human β_2m gene) were then crossed and their progeny analyzed by Southern blotting (Figure 1). Both transgenes segregated in a Mendelian fashion and from 13 offspring, 3 animals were obtained that had received both transgenes.

The tissue specific expression of both transgenes was examined by extraction of RNA from different tissues of mouse 83, transgenic for both genes, followed by Northern blot analysis (Figure 2). Those tissues which are known to be strongly positive for Class I antigens in mouse (Klein, 1986) also revealed strong signals on Northern blot analysis for both HLA-B27 heavy chain and human β_2m mRNA. Thus the regulation of expression of both transgenes seems to follow that of the endogenous, murine Class I genes. This supposition was indeed confirmed by com-

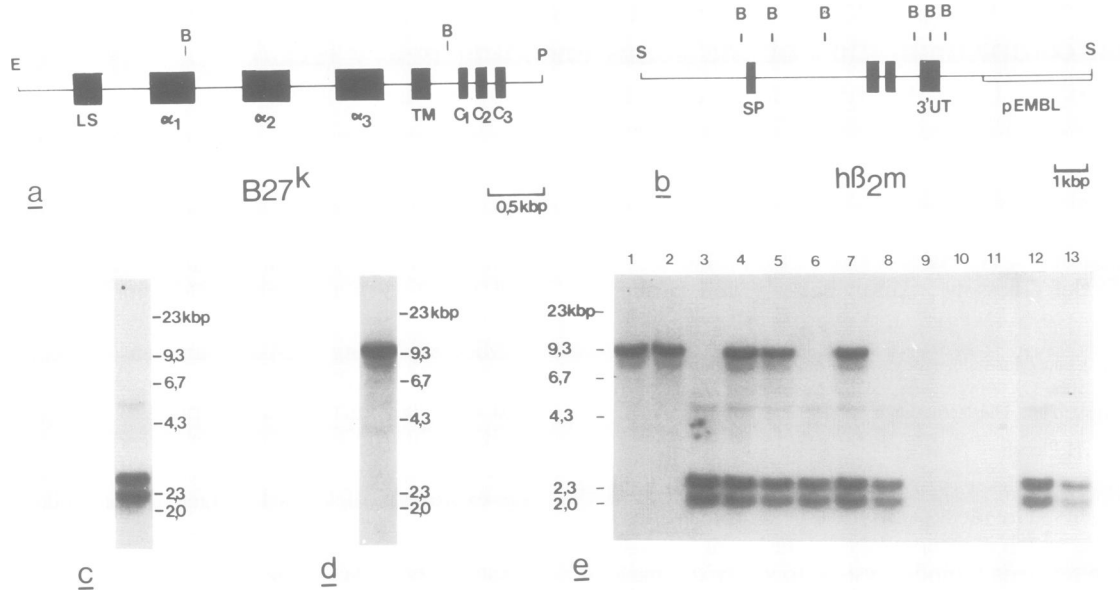

Fig. 1. DNA analysis of transgenic mice and their offspring. **(a)** Schematic representation of the HLA-B27 gene used for injection. Solid bars represent exon sequences; *Bgl*II, *Eco*RI and *Pst*I restriction sites are indicated as B, E and P, respectively; for further details see Seemann *et al.*, 1986. **(b)** Schematic representation of the human β_2m gene construct; solid bars represent exon sequences (Guessow, in preparation); open bar represents bacterial vector sequences (pEMBL9); *Bgl*II and *Sal*I sites are indicated as B and S respectively. **(c)** Detection of HLA-B27 sequences in total genomic DNA of the transgenic mouse 22 used in the cross shown in panel e. 8 μg of chromosomal DNA, isolated from a tail biopsy, was digested with *Bgl*II and analyzed by gel electrophoresis and Southern blotting using an HLA-B7 cDNA as a probe (Sood *et al.*, 1981). In a tandem array of HLA-B27 gene copies, *Bgl*II generates two hybridizing fragments of 2.1 and 2.4 kbp. The results of this analysis indicate the integration of multiple copies of HLA-B27 in mouse 22, but the actual number has not been determined. **(d)** Detection of the human β_2m gene construct in total genomic DNA in mouse 55, used in the cross shown in panel e. Chromosomal DNA was analyzed as described in c, but using pEMBL9 as a probe. In a concatemer of human β_2m constructs, *Bgl*II creates a hybridizing fragment of 10.5 kbp spanning the junction of two tandemly arranged copies. The results indicate that mouse 55 indeed carried multiple copies of the human β_2m construct. **(e)** DNA analysis of offspring from a cross between transgenic mouse 22 (HLA-B27) an mouse 55 (human β_2m). Chromosomal DNA was digested with *Bgl*II and analyzed as described in c, using the HLA-B7 cDNA and pEMBL9 as probes. Both transgenes are transmitted to offspring in a Mendelian fashion (HLA-B27 alone: 5/13; human β_2m alone: 2/13; both HLA-B27 and human β_2m: 3/13; none: 3/13).

paring the levels of HLA mRNAs to those of mouse H-2 and β_2m mRNAs (data not shown).

Biochemical analysis of protein expression

Crosses between HLA-B27 and human β_2m transgenic mice revealed three offspring carrying both genes, from which splenocytes were isolated for protein characterization; littermates carrying no transgenes, and animals positive for only the HLA-B27 or the human β_2m gene served as controls.

Analysis of the protein products by immunoprecipitation was carried out using (i) the monoclonal antibody (Mab) HC10 that reacts exclusively with free HLA Class I heavy chains (Stam *et al.*, 1986), (ii) the Mab W6/32 that reacts exclusively with the Class I heavy chain — β_2m complex (Parham *et al.*, 1979) and that reacts poorly with hybrid molecules consisting of human Class I heavy chain — mouse β_2m (Ferrier, 1985) and (iii) the anti-human β_2m Mab BBM1 (Brodsky *et al.*, 1979). As controls, immunoprecipitations with rabbit anti-mouse β_2m and anti-H-2K, D antibodies were carried out. The immunoprecipitated antigens were then analyzed by one-dimensional isoelectric focusing (1D-IEF), a technique that readily resolves all of the polypeptide chains and post-translational modifications relevant to these experiment (Neefjes *et al.*, 1986; Tulp *et al.*, 1986).

The results shown in Figure 3 establish that mice having received the human β_2m gene not only produce free human β_2m, but also that hybrid antigens consisting of murine Class I heavy chains in association with human β_2m are demonstrable. This is in contrast with the apparent inability of the HLA-B27 heavy chain to engage in efficient complex formation with mouse β_2m (Rein *et al.*, 1987). Mice carrying only the HLA-B27 gene produced considerable quantities of free HLA-B27 heavy chains, but no

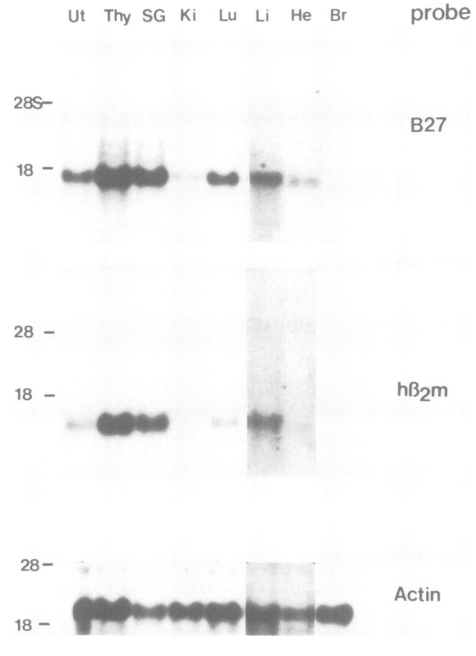

Fig. 2. Detection of HLA-B27 and human β_2m mRNA in a mouse transgenic for HLA-B27 and human β_2m. Northern blot analysis of total RNA isolated from several tissues of transgenic mouse 83, carrying both HLA-B27 and human β_2m genes. 15 μg of total cellular RNA was electrophoresed on a 1% agarose gel, transferred to nitrocellulose and probed with an HLA-B7 cDNA probe, a human β_2m cDNA probe and an actin-specific probe to show quantity and integrity of the RNA. Ut: uterus; Thy: thymus; SG: salivary gland; Ki: kidney; Lu: lung; Li: liver; He: heart; Br: brain.

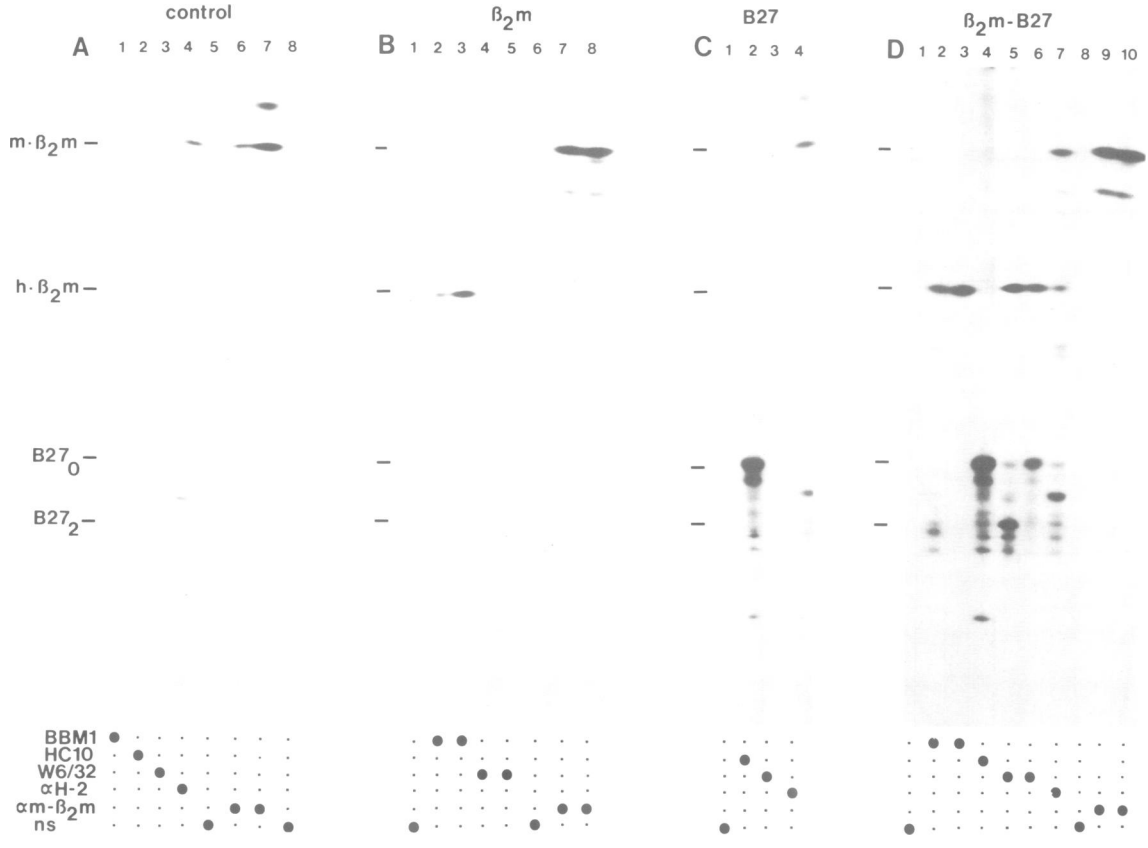

Fig. 3. Biochemical analysis of Class I antigens transgenic mice. Splenocytes from mice were radiolabeled with [^{35}S]methionine and analyzed for Class I antigens by immunoprecipitation. The resulting immunoprecipitates were analyzed by 1D-IEF, either directly or after treatment with neuraminidase (NANAse). The position of migration of mouse β_2m (mβ2m), human β_2m (hβ2m), HLA-B27 heavy chain carrying no ($B27_0$) or two ($B27_2$) sialic acid residues are indicated. The reagents used for immunoprecipitation are indicated below each panel. **Panel A:** control mouse derived from the cross shown in Figure 1e having received neither the HLA-B27 nor the human β_2m gene. The antihuman Class I reagents do not cross-react with mouse Class I antigens. Lane 1: BBM1; lane 2: HC10; lane 3 W6/32; lane 4: H-2 (the H-2 Class I heavy chains migrating between $B27_0$ and $B27_2$ have not been further identified); lane 5: normal mouse serum; Lane 6: αm-β_2m, precipitation on splenocyte culture supernate; lane 7: as lane 6, but splenocyte lysate was used; lane 8: normal rabbit serum. **Panel B:** Human β2m transgenic mouse. The expression of human β_2m but not HLA-B27 heavy chain is demonstrable. Lane 1: normal mouse serum; lane 2: BBM1, splenocyte lysate; lane 3: BBM1, culture supernate; lane 4: W6/32; lane 5: W6/32, NANAse treated; lane 6: normal rabbit serum; lane 7: αm-β_2m, splenocyte supernate; lane 8: as lane 7, splenocyte lyate. **Panel C:** HLA-B27 transgenic mouse. Synthesis of HLA-B27 heavy chains is demonstrable but no assembled W6/32 reactive complexes can be detected. Lane 1: normal mouse serum; lane 2: HC10; lane 3: W6/32; lane 4: αH-2. **Panel D:** Human β_2m-HLA-B27 transgenic mouse. Fully assembled HLA-B27 antigens carrying sialic acids are detectable. Lane 1: normal mouse serum; lane 2: BBM1, splenocyte lysate; lane 3: BBM1, splenocyte culture supernate; lane 4: HC10; lane 5 W6/32; lane 6: W6/32, NANAse treated; lane 7: αH-2 (note the presence of both human and mouse β_2m, indicating the formation of hybrid H-2 antigens); lane 8 normal rabbit serum; lane 9: αm-β_2m, splenocyte culture supernatant; lane 10: αm-β_2m, splenocyte lysate.

assembled HLA-B27 antigen can be recovered with the W6/32 antibody (Figure 3C), as was also found in studies involving transfected murine cell lines. Somewhat surprisingly, rabbit anti-mouse β_2m was evidently unable to immunoprecipitate murine β_2m associated with either murine or human Class I heavy chains (Figure 3a,b,c). In those mice that had received both transgenes, correctly assembled HLA-B27 could be isolated using the W6/32 antibody (Figure 3d). The presence of human β_2m in immunoprecipitates with the anti-H-2 reagents is clearly apparent (Figure 3d, lane 7) in these animals.

Surface Class I molecules invariably carry sialic acids on their N-linked glycan (Ploegh *et al.*, 1981), the presence of which may be taken as a reliable indicator of correct assembly, transport and delivery to the cell surface of Class I antigens. Mice carrying both transgenes indeed produced fully modified, i.e. neuraminidase-sensitive, HLA-B27 antigens (Figure 3d, lanes 5,6).

The level of HLA-B27 expression observed in these animals are comparable to those observed for the H-2 antigens used as controls. This holds true for both subunits, where it should also be born in mind that mouse β_2m contains five and human β_2m one methionine residue per molecule (Ploegh *et al.*, 1981).

FACS analysis of protein expression on splenocytes

Splenocytes were obtained from mice derived from the cross shown in Figure 1e and analyzed by staining with FITC-conjugated W6/32 and cytofluorimetry. The results were fully consistent with the biochemical analysis (Figure 4). Only cells from mice having received both transgenes could be stained with the W6/32 antibody. When comparing the relative fluorescence intensity of splenocytes from transgenic mice with human peripheral blood lymphocytes (Figure 4, bottom panel), one should take into account that the transgenic animal expresses only a single HLA allele, whereas any human lymphocyte will obviously express both alleles at the HLA-A, -B, and -C loci, all of which react with W6/32 (Parham *et al.*, 1979).

The results reported here demonstrate proper cell surface expression of human histocompatibility antigens in transgenic mice. This result was obtained by crossing two independently derived transgenic mouse strains. Many gene products require assembly into heterodimers in order to be functionally active, and application of transgenic technology to the study of regulation of expression of such genes will therefore require the simultaneous

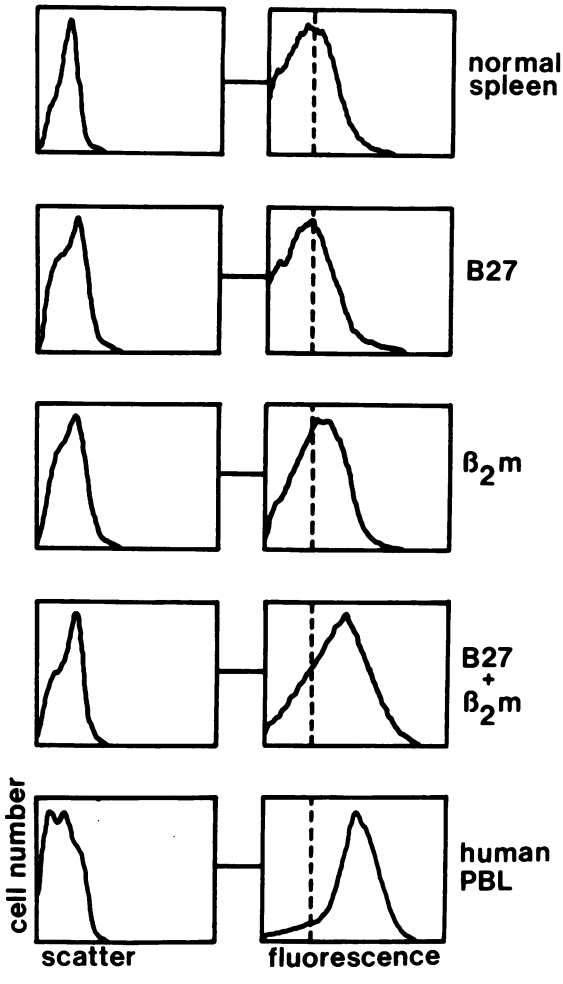

Fig. 4. Cytofluorimetric analysis of splenocytes from transgenic mice. Splenocytes were obtained from control mice derived from the cross shown in Figure 1e, having received neither the HLA-B27 nor the numan β_2m gene (normal spleen), from mice carrying either transgene alone (β_2m or B27 respectively), or in combination (B27 + β_2m), as indicated on the right of the figure. Splenocytes were analyzed after staining with W6/32 directly conjugated with fluoresceinisothiocyanate. For comparison, a similar analysis was carried out on human peripheral blood lymphocytes (human PBL; bottom panel). The left panels show the forward light scatter diagrams, the right panels show the fluorescence expressed in arbitrary units. The dashed line indicates the peak channel of fluorescence intensity observed for the normal mouse splenocytes.

or consecutive introduction of the relevant genes. We have shown here that crosses between transgenic animals can indeed be used successfully to achieve this goal.

Materials and methods

Transgenic mice

A 4,3 kb *Eco*RI fragment was isolated from the HLA-B27 genomic clone (the HLA-B27 subtype was used, Seemann *et al.*, 1986) after subcloning into pUC18, by preparative gel electrophoresis and electroelution. The pEMBL plasmid harboring the human β_2m gene (Guessow *et al.*, unpublished observations, Rein *et al.*, 1987) was linearized with *Sal*I; bacterial vector sequences were not removed.

Fertilized mouse eggs were recovered in cumulus from the oviducts of superovulated (CBA × C57Bl/LiA) F1 females that had mated with F1 males several hours earlier. Approximately 400 copies of either the HLA-B27 of the human β_2m gene were microinjected in the most accessible pronucleus of each fertilized egg. Microinjected eggs were implanted into the oviducts of 1-day pseudopregnant MA or F1 foster mothers and carried to term. Several weeks after birth of animals that had developed from microinjected eggs, total genomic DNA was prepared from tail biopsies. For Southern blot analysis, 8 μg of total genomic DNA of each mouse was digested with *Bgl*II, run on a 0.6% agarose gel and transferred to nitrocellulose. The filter was hybridized to ^{32}P-labeled probes and washed as described (Cuypers *et al.*, 1984).

Northern blot analysis

Fifteen μg of total cellular RNA, prepared by the LiCl—urea method (Auffray and Rougeon, 1980) was electrophoresed on a 1% agarose formaldehyde gel (Selten *et al.*, 1984), transferred to nitrocellulose and probed with an HLA-B27 probe, a human β_2m cDNA probe and an actin-specific probe (Dodemont *et al.*, 1982) to show quantity and integrity of the RNA. The final wash of the blot was carried out for 15 min. at 65°C in 0.1× standard saline citrate.

Biochemical analysis of Class I antigens

Splenocytes from mice were radiolabeled with [^{35}S]methionine and Class I antigens were isolated by immunoprecipitation as described for human lymphoid cells (Tulp, 1986; Neefjes *et al.*, 1986). The following reagents were used: BBM1, an anti-human β_2m Mab that does not react with heavy chain —β_2m complexes (Stam *et al.*, 1986); W6/32, an anti-HLA-A, -B, -C antibody that recognizes only fully assembled Class I molecules (Parham *et al.*, 1979); H-2, a mixture of the following mouse Mabs: F5.21.27 (m17), 20.8.4 (m57), 28.8.6 (m61) and H142-45 (m48) specific for the H-2q and H-2b haplotypes, kindly provided by Prof.J.Klein, Tuebingen; α-β_2m, a rabbit anti mouse β_2m serum kindly provided by Dr P.Peterson, Uppsala, a reagent which reacts preferentially with free mouse β_2m; normal serum from either mouse or rabbit, as indicated in the figure legend. The resulting immunoprecipitates, all prepared from equal aliquots of lysate, were analyzed by 1D-IEF (Tulp, 1986; Neefjes *et al.*, 1986), either directly or after treatment with neuraminidase (NANAse) as described (Neefjes *et al.*, 1986).

Cytofluorimetric analysis of splenocytes

Splenocytes were stained with h.p.l.c.-purified W6/32 antibody which had been directly conjugated with fluoresceinisothiocyanate, and analyzed in a FACS IV (Becton-Dickinson).

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