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ARTICLES

β2-Microglobulin deficient mice lack CD4\(^{-8}\) cytolytic T cells

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Mice homozygous for a β2-microglobulin gene disruption do not express any detectable β2-m protein. They express little if any functional major histocompatibility complex (MHC) class I antigen on the cell surface yet are fertile and apparently healthy. They show a normal distribution of γδ, CD4\(^{\text{+}}\) and CD4\(^{\text{-8}}\) T cells, but have no mature CD4\(^{\text{+}}\) T cells and are defective in CD4\(^{\text{-8}}\) T cell-mediated cytotoxicity. Our results strongly support earlier evidence that MHC class I molecules are crucial for positive selection of T cell antigen receptor αβ in CD4\(^{\text{-8}}\) T cells in the thymus and call into question the non-immune functions that have been ascribed to MHC class I molecules.

β2-MICROGLOBULIN (β2-m) is a polypeptide of relative molecular mass 12,000 (12K), which is activated in mouse embryos by the 2-cell stage, and associates with the heavy chain of the polymorphic MHC class I proteins encoded by the H2-K, H2-L/D and Qa/Tla loci. The role of MHC class I proteins in the presentation of antigens to the immune system and in the development of the T-cell repertoire is well documented, but it has been suggested that MHC molecules have other non-immunological functions, for example, as differentiation antigens or in the function of hormone receptors or as olfactory cues influencing mating behaviour. Furthermore, it has been demonstrated that β2-m associates with the Fc receptor in neonatal gut cells, induces collagenase activity in fibroblasts, and may serve as a chetomactic protein in the fetal thymus. We and others have disrupted the β2-m gene by targeted mutation, and generated a mutant mouse strain that is unable to express β2-m protein and therefore most class I molecules, which require β2-m for assembly and cell surface expression.

Normal development

Mice heterozygous for the disrupted β2-m gene were intercrossed to derive animals homozygous for the mutation. DNA was isolated from embryos between day 14 and 18 of gestation, or from the tails of weaning mice, and the genotype of each animal was determined by Southern blot analysis as described. Table 1 shows that of 33 embryos and 23 of 101 adults were homozygous for the mutated β2-m gene. Homozygotes were indistinguishable from heterozygous or wild-type littermates, had normal body weight and, on autopsy, showed no noticeable alterations in any organ. When bred, normal sized litters were born and raised by homozygous parents. These results indicate that the mutation has no obviously detrimental effect on the well-being or breeding performance of the animals.

Truncated β2-m mRNA

Transcription of the wild-type (+/+ ) β2-m gene results in two messenger RNA species of 0.8 and 1.0 kilobases (kb) that are due to the use of alternative polyadenylation signals in exon 4 (Fig. 1a, ref. 21). The mutant β2-m gene contains a 1.1-kb fragment of plasmid pMCINEO inserted into exon 2 which is transcribed from the tk promoter. The inserted neo gene has the same transcriptional orientation as the disrupted β2-m gene and lacks a polyadenylation signal.

To characterize class I-specific mRNA in mutant mice, total RNA was prepared from tissues of +/+ , +/− (heterozygous) and −/− (homozygous) mice or from embryonic fibroblasts and examined by northern blot analysis. The blots were hybridized to a β2-m probe, the neo probe and an MHC class I-heavy chain probe. Figure 1b shows two strong bands at 0.8 and 1.0 kb expected for β2-m mRNA in interferon-γ treated +/+ and +/− fibroblasts, whereas untreated cells showed a much weaker signal. In contrast, homozygous mutant cells did not synthesize the normal β2-m mRNA species, but showed instead a band at 2.0 kb which was also seen in +/− cells, and is expected for an RNA species initiated at the β2-m promoter, transcribed through the neo cassette and terminated in exon 4. The intensity of the signal suggested that this RNA was much less abundant than the wild-type β2-m RNA. Similar hybridization signals were detected in liver, kidney, spleen, brain and lung RNA of adult mutant or wild-type mice (Fig. 1c, and data not shown). In addition, a faint signal of 1.5 kb was seen in +/− and −/− animals, which may correspond to RNA initiated at the tk promoter.

We do not know why transcription from the β2-m promoter is impaired in cells of homozygous mice. It is possible that the neo insert exerts an inhibitory effect on β2-m promoter usage or that the read-through RNA is less stable. Alternatively, additional mutations not detectable by Southern analysis may have occurred in the promoter region and interfere with its function. Hybriization to the MHC class I heavy chain probe showed the expected signal of 1.6 kb with the same intensity in animals of all three genotypes (Fig. 1b, c). The results shown in Fig. 1 indicate that the β2-m gene disruption prevents synthesis of normal β2-m RNA but does not interfere with the transcription or stability of MHC class I-heavy chain RNA.

No β2-m protein

Embryonic fibroblasts were treated with interferon-γ, labelled with \(^{[35]}\)S\) methionine and protein extracts subjected to immune precipitation using β2-m and several MHC class I-specific antibodies. Figure 2a shows that the expected 12K protein was detected by precipitation with the β2-m specific antiserum in +/+ and +/− cells, but was absent in −/− cells. Recognition of the H-2K\(^{\text{b}}\) heavy chain by monoclonal antibody 5F1.1.24 (anti H-2\(^{\text{K}}\), α2 domain) was dependent on association of the heavy chain with β2-m because the expected 46K band was seen in wild-type and heterozygous cells but was not detected in mutant cells. In contrast, immunoprecipitation with monoclonal antibody 28-14-85 (anti H-2\(^{\text{D}}\), α3 domain) precipitated 44K and 46K heavy chain bands and the 12K β2-m band in wild-type and heterozygous cells, as well as a weaker 44K band in homozygous cells. The 44K molecule most probably represents an immature precursor molecule still containing the terminal high...
mice with any of the β2-m, H-2Kb and Qa-2 specific antibodies. However, incubation with several different anti-D7 monoclonal antibodies resulted in detectable staining which was reduced 20-fold or more when compared with wild-type cells. This observation corroborates the notion that the H-2Dd molecule can reach the cell surface even in the absence of endogenous β2-m. The native configuration of D7 as detected by these antibodies may, however, depend on exogenous β2-m derived from the culture medium. Results of a preliminary staining experiment performed in serum-free medium suggest that decreased D7-specific staining occurs in the absence of exogenous β2-m.

**MHC class I cell surface expression**

Surface expression of MHC class I molecules was examined by incubating purified CD4+8+ T cells with a panel of MHC class I specific antibodies and evaluated by fluorescence-activated cell sorting (FACS) analyses. The data shown in Fig. 2B and Table 2 failed to reveal any staining of cells from homozygous

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**FIG. 1.** Expression of β2-m. a, Structure of the wild-type (wt) and mutant β2-m genes and the predicted sizes of correctly spliced β2-m and/or neo-specific mRNA transcripts. A 350-base pair (bp) PstI-EcoRI fragment of β2-m complementary DNA was used as a β2-m-specific hybridization probe. This fragment spans the 5′ start site in exon 1 to the EcoRI site in exon 2. The following fragments were also used as hybridization probes: a 910-bp EcoRI-EcoRI fragment derived from MHC class I heavy chain probe; a 1.4-kb PstI fragment from pJ2F-37 containing a complete DNA of H-2Kd gene; MHC class I heavy chain probe; and a 1.4-kb PstI fragment containing the entire rat α-tubulin cDNA (tubulin probe). Northern blot analysis of 15 μg total cellular RNA from primary fibroblasts derived from 14-day-old F2 embryos of indicated genotype. Cells were grown in MEM supplemented with 10% FCS, 1x non-essential amino-acids (Gibco). Interferon treatment was performed for 24 hours by supplementing the medium with 500 U/ml recombinant mouse IFN-γ (Genzyme). b, Northern blot analysis of 15 μg total cellular RNA or 0.5 μg oligo(dT)-selected poly(A)+ mRNA (one cycle) derived from adult liver and kidney of F2 animals of indicated genotypes. Total cellular RNA was isolated by the acid guanidinium method and separated on a 1.5% formaldehyde-treated agarose gel and blot-hybridized with standard procedures. "Bars were stripped of hybridizing probes by treatment for 20 min in 10 mM Tris buffer, pH 7.5, 0.1% SDS, at 80°C before reprobing. Bars indicate the migration of the 18S ribosomal RNA.

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**ARTICLES**

**No functional Fe receptor**

Recently, the β2-m protein has been identified as the smaller component of the Fe receptor that mediates the uptake of IgG from milk in intestinal cells of neonatal rats. To examine the role of β2-m for the biological function of the mouse intestinal Fe receptor, brush borders were isolated from the small intestine of 11-day-old littermates and tested for the binding of [125I]-labelled IgG. The results demonstrate that intestinal cells from homozygous mutant mice fail to show significant binding of IgG (0.6%±1.3 (mean±s.d.) of total c.p.m. bound; n=4). The amount of IgG bound to brush borders from heterozygotes (0.2%±1.9; n=4) and wild-type mice (6.9%±3.3; n=2) was indistinguishable. This indicates that the Fe receptor heavy chain in mouse must associate with β2-m protein for functional expression on the cell surface.

**No TCR αβ+ CD4+8+ T Cells**

Lymphoid organs derived from F2 animals of the three genotypes were characterized by two- and three-colour FACS analyses for the presence of different subtypes of T cells. In both the adult thymus and spleen of 11-day-old adult homozygous mutant mice, a dramatic 100-150-fold reduction in T-cell antigen receptor (TCR) αβ+ CD4+8+ T cells was observed (Fig. 3, and data not shown). No differences between heterozygotes and wild-type T cells were seen (data not shown). In the thymus of
homzygous mutant mice the presence of the populations of TCR αβCD4+CD8+ and αβCD4-CD8+ T cells was unaltered (TCH staining not shown). Also, αβ-CD4-CD8+ thymocytes were present in normal numbers in the thymus of young (day 11) mice. These cells are thought to represent an intermediate between αβ-CD4+CD8+ and αβ-CD4+CD8+ T cell lineages (25–29). Therefore, our results imply that MHC class I cell-surface expression is only essential for the development of the TCR αβCD4+CD8+ T cells. This indicates that differentiation of αβCD4+CD8+ T cells from αβ-CD4+CD8+ thymocytes requires interaction with class I MHC molecules. Finally, it should be noted that the presence of the MHC class II-restricted CD4+CD8+ T cells (Fig. 3), and surface IgB cells (data not shown) are unchanged in the homozygous mutant mice.

Presence of γδ+ T cells unaffected

There is evidence that at least a portion of TCR γδ-γδ+ T cells may be restricted by MHC class I-like molecules encoded by the Qa-TL region of the H-2 complex (26–28). Therefore thymocytes obtained from 17.5-day-old embryos and thymus and spleen from adult mice were examined for the presence of γδ+ T cells. The number of γδ+ cells was not significantly affected by the β2-mutation (Fig. 3g, h). Furthermore, the abundance in the fetal (E17.5) thymus of a subset of γδ+ cells expressing the Vγ6

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**TABLE 1** Transmission of mutant β2-m gene

<table>
<thead>
<tr>
<th>Parents</th>
<th>No. of Litters (age)</th>
<th>Genotype of progeny (no. of)</th>
</tr>
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<tbody>
<tr>
<td>+/− x +/−</td>
<td>4 (embryonic)</td>
<td>9 17 7</td>
</tr>
<tr>
<td>−/− x −/−</td>
<td>2 (neonatal)</td>
<td>23 52 26</td>
</tr>
</tbody>
</table>

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**TABLE 2** Cell surface expression of MHC class I molecules

<table>
<thead>
<tr>
<th>1st Reagent</th>
<th>Specificity</th>
<th>Mean Fluorescence Intensity</th>
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</thead>
<tbody>
<tr>
<td>none</td>
<td>−</td>
<td>1.7</td>
</tr>
<tr>
<td>NE-209</td>
<td>β2-m</td>
<td>168.2</td>
</tr>
<tr>
<td>M1/42.3.8</td>
<td>αβ CD4+</td>
<td>347.7</td>
</tr>
<tr>
<td>H-2Kd</td>
<td>αβ CD4+</td>
<td>219.4</td>
</tr>
<tr>
<td>K10.56-1</td>
<td>αβ CD4-CD8+</td>
<td>8.2</td>
</tr>
<tr>
<td>K7-300</td>
<td>αβ CD4-CD8+</td>
<td>6.7</td>
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<td>BB-24.3</td>
<td>αβ CD4-CD8+</td>
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<td>139.5</td>
</tr>
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<td>5F1.1.24</td>
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<td>63.2</td>
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<tr>
<td>2B-14-85</td>
<td>αβ CD4-CD8+</td>
<td>119.3</td>
</tr>
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<td>αβ CD4-CD8+</td>
<td>36.0</td>
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<td>27-11-13S</td>
<td>αβ CD4-CD8+</td>
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<td>D3-262</td>
<td>αβ CD4-CD8+</td>
<td>38.6</td>
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<td>1-4-9</td>
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<tr>
<td>1-4-4</td>
<td>αβ CD4-CD8+</td>
<td>29.6</td>
</tr>
</tbody>
</table>

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**FIG. 2** Expression of MHC class I molecules: a, Immunoprecipitations of MHC class I molecules from metabolically labelled embryonic fibroblasts. Fibroblasts derived from day-14 embryos were routinely cultured (see Fig. 1b). Normal medium was supplemented with 500 μM iodoacetic acid for 1 h. After washing with medium containing 9 volumes of PBS to 1 volume normal DMEM supplemented with 5% dialyzed FCS, the medium was added to 2.5 ml of medium containing 0.2 M iodoacetic acid (25). Subsequently, cells were incubated for 1 h at 37°C in normal DMEM. The supernatant was removed and washed with PBS and resuspended in lysis buffer. The supernatant was then solubilized in the presence of 10% SDS, 1% β-mercaptoethanol, and 1% sodium dodecyl sulfate (SDS) and SDS-PAGE. b, Cell surface expression of MHC class I molecules from purified T cells obtained from lymph nodes of 4-week-old wild-type (+/+) and homozygous mutant (−/−) F2 animals. T cells from 5-week-old H-2B-IB10.BR mice served as negative controls for staining. 

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**FIG. 3** The presence of γδ+ T cells in normal (a, b, e) and homozygous mutant (c, d, f) mice. At birth, the γδ+ T cells constituted approximately 1% of the total T cell population. In the absence of β2-m, the number of γδ+ T cells decreased to less than 0.1% of the total T cell population. The proportion of γδ+ T cells in the thymus was significantly lower in homozygous mutant mice than in normal mice. In contrast, the number of γδ+ T cells in the spleen was not significantly different between normal and homozygous mutant mice. The percentage of γδ+ T cells in the peripheral blood was also similar in normal and homozygous mutant mice.
against completely allogeneic BALB/c (H-2\(^b\)) cells. Spleen cells derived from congenitally mutant mice were virtually devoid of any CTL-p (Fig. 4c). The CTL responses of heterozygous animals were indistinguishable from those obtained with wild-type animals (data not shown). In addition, we examined whether the mutant cells could stimulate CTL-p or serve as target cells for established CTL. As shown in Fig. 4a, β2-m-negative spleen cells fail to elicit a significant CTL response by BALB/c responders. Similar results were obtained with B10.BR responders (data not shown). Therefore the low residual H-2\(^{D\ast}\) cells surface expression in homozygous mutant cells is clearly not sufficient to trigger a vigorous CTL response.

Finally we find that mutant cells can serve as target cells for anti-H-2\(^b\) CTL generated in conventional ML, although about

ninefold more CTL are required to lyse mutant compared with wild-type targets (Fig. 4b). This residual killing can be accounted for by three hypotheses. First, it is possible that D\(^{\ast}\) molecules assume a functional conformation even in the absence of β2-m, albeit at a dramatically reduced level. Alternatively, bovine β2-m from the serum-containing medium may associate with cell surface D\(^{\ast}\) and facilitate refolding of the molecule. This latter hypothesis is consistent with published data demonstrating the binding of serum β2-m to class I molecules on cultured cells. Finally, it is possible that the residual CTL activity is specific for non-class I antigens, although no lysis of B10.BR targets was observed (Fig. 4b) and highly purified mutant T-cell blasts

FIG. 3. Analysis of T-cell subsets in wild-type (+/+) and homozygous mutant (−/−) mice. Splenocytes (a, b) and thymocytes (c, d) from 13-day-old mice were stained with anti-CD4-phycocerythin and anti-CD8-PE/CY5 (Becton and Dickinson, Mountainview, California). In panels a, h CD8\(^+\)/CD4\(^-\) cells and y cells were first enriched from thymocyte populations of 4-5-week-old mice by eliminating CD4\(^+\) cells (most y cells are CD4\(^-\)/CD8\(^+\)). The enriched cells were stained with anti-μβ TCR monoclonal antibody (HST-597\(^b\)T; e, f), or anti-γ1 TCR monoclonal antibody (TCT-13D5; e, f). Bluestone, unpublished data) (g, h) (ordinate), and with anti-CD8 antibody (a, h) (abscissa). The percentages of the cells in relevant quadrants are indicated on the figure. In e and g, the lack of CD8\(^+\) cells leads to a twofold enrichment of γ cells in several experiments there was no obvious difference between +/+ and −/− mice in the frequency of CD8\(^-\) cells that are γ positive. In addition, the presence of γ cells was examined in unfraccionated thymocytes of day-17.5 embryos. No significant differences were seen in the number of cells reactive with anti-γ1 antibody (C/T-13D5; −/−, 1.83 ± 0.27 (n = 6); +/+, 1.99 ± 0.03 (n = 5)). Also, no changes in the number of cells reactive with antibody F-536 (anti-V\(^\gamma\),4) were noted; −/−, 0.88 ± 0.22 (n = 6); +/+, 0.80 ± 0.03 (n = 3) and +/+, 1.10 ± 0.04 (n = 5).

METHODS. To eliminate CD4\(^+\) cells, thymocytes were incubated with anti-CD4 (L344.5) monoclonal antibody and complement for 40 min. Viable cells were purified on Ficoll-isopaque gradients and subjected to a second round of killing with mouse anti-rat antibody (MAR18.5) antibody plus complement to eliminate residual CD4\(^+\) cells with bound GK1.5. The viable cells were again purified on Ficoll-isopaque gradients. To stain β2 TCR versus CD8, the enriched cells were reacted with HST-597-biotin followed in a second step with phycoerythrin-streptavidin (Becton and Dickinson) and anti-CD8-PE/CY5. To stain γ TCR versus CD8, enriched cells were first reacted with UTC-13D5 culture supernatant, followed in a second step by goat-anti-hamster IgG-phycocerythrin (reagent adsorbed with rat and mouse IgG, Caltag, California). After washing, the cells were incubated with rat-FIG, to ensure that there were no free rat immunoglobulin-binding sites, and subsequently reacted with anti-CD8-PE/CY5. In all cases the negative controls using all reagents except the TCR-specific first reagents, generated insignificant numbers of positive cells. Curators were set based on the negative control samples. Dead cells were excluded based on forward and 90°-degree light scatter characteristics. One hundred thousand cells were analysed on a FACSCALF (a-f) or 3 × 10\(^4\) cells on an EPICS C (g, h) flow cytometer.

FIG. 4. Functional studies of β2-m mutant mice. a, β2-m mutant (−/−) F2 spleen cells (a, c, d) from two animals stimulate little or no BALB/c (H-2\(^b\)) cytotoxic T lymphocytes (CTL) reactive with H-2\(^b\). Shown for comparison are cultures stimulated with wild-type littermate (+/+) (b, c, d) and syngenic spleen cells (e). All cultures were tested against C57BL/6 (H-2\(^b\)) target cells. b, β2-m mutant (−/−) concanavalin A-induced blast cells (c) serve as targets for conventional H-2\(^b\) CTL. The CTL were from secondary mixed lymphocyte culture of B10BR spleen cells stimulated with wild-type (+/+) (d). Spleen cells showed for comparison is the lytic activity of wild-type littermate target cells (b) and B10.BR target cells (d). Approximately nine times more of the CTL are required to achieve the same level of lysis of β2-m\(^−\) targets as β2-m\(^−\) targets. c, Spleen cells of two β2-m mutant (−/−) mice (c, d) fail to generate allo-specific CTL when stimulated with BALB/c (H-2\(^b\)) spleen cells. Shown for comparison are the responses of two wild-type littermates (a, b). Cultures were tested on BALB/c or congenital A-induced target cells. d, β2-m mutant (−/−) lymph node proliferate in response to alloantigen (BALB/c, H-2\(^b\)) stimulator cells (hatched bars). The response of a wild-type littermate is shown for comparison (solid bars). Cultures stimulated with syngenic or C57BL/6 spleen cells served as negative controls. Proliferative response was determined between days 5-6 of culture by pulse with [3H]thymidine (0.5 μCi). METHODS. Details of mixed lymphocyte culture and T-cell assay procedures are described. In all cases target cells were from cultures of spleen cells incubated for 2 days with 2 μg m\(^{-1}\) concanavalin A and labelled for 1 h with 200 μCi 31P. CTL assay was for 4 h. MLR (a, d) was performed in round-bottom microtitre wells with 1 × 10\(^5\) responder lymph node cells and 5 × 10\(^5\) irradiated (3000 rads) spleen cells stimulator.
are lysed, arguing that the activity is not directed against class II molecules (data not shown).

When mutant cells were used as either responders (Fig. 4d) or as stimulator cells (data not shown) in a mixed lymphocyte reaction (MLR) the proliferative responses were similar to those with wild-type cells. This is consistent with the fact that the proliferation measured in an MLR is predominantly determined by the recognition of foreign MHC class II molecules by CD4+ T cells

**Discussion**

Mice homozygous for the β2-m gene disruption develop normally in the absence of detectable β2-m protein. Our results therefore question the widely held view that class I molecules have an essential role in the embryonic development of vertebrates, although the possibility that some class I molecules may be functional in the absence of β2-m has not been ruled out. That mutant embryos have a normal sized thymus argues that the function of β2-m as a chemotactic protein (thymotaxin) is not essential for thymus development.

Of interest is the apparently normal development of γδ T cells in β2-m mutant mice. The physiological functions and specificity of γδ T cells are not yet understood, but it has been suggested that γδ T cells functionally interact with class I molecules, particularly those encoded by Qa2/TL region genes telomeric to the H-2 complex. If γδ T cells naturally interact with class I molecules, it might be expected that like CD8+ T cells, interactions with class I molecules would be required for their differentiation. Although our data argue against this, it is still possible that only mature γδ T cells recognize class I molecules or antigens associated with class I molecules. In addition, it is possible that differentiation of only a subset of γδ T cells requires class I MHC molecules.

The most striking phenotype of the β2-m mutant mice is the virtually complete absence of the mature αβ CD4+ CD8+ T cell subset, in both the thymus and peripheral lymphoid organs. These data argue that encounters with class I MHC molecules are essential for the differentiation of the αβ CD4+ CD8+ T cell subset, consistent with the conclusions drawn from studies of transgenic mice expressing a defined αβ TCR in all their T cells

As expected from the absence of mature CD4+ T cells, the CTL responses in homozygous mutant mice were abolished. The animals, when kept under pathogen-free conditions, appear to be healthy. It will, however, be interesting to study the role of MHC class I antigens in the response to viral or other infections or in tumorigenesis by exposing the animals to infectious agents or to carcinogens. Also, because of the deficiency in class I-dependent functions, homozygous mutant mice may serve as donors or recipients in transplantation experiments across histocompatibility barriers without treating the recipients with immunosuppressive regimens. Finally, transplantations of mutant bone marrow cells to normal mice should test the hypothesis that rejection of bone marrow (haemopoietic histocompatibility) can depend on the failure of marrow cells to express self class I molecules.

These studies will be greatly aided by the availability of a homozygous breeding colony.