An Impaired Breeding Phenotype in Mice with a Genetic Deletion of Beta-2 Microglobulin and Diminished MHC Class I Expression: Role in Reproductive Fitness[1]

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ABSTRACT

Beta-2 microglobulin (B2M) plays a pivotal role in the biology of mammals, including its association with major histocompatibility complex (MHC) Class I gene products. The latter molecules have been shown to affect reproduction in both mice and humans, although the exact mechanism is still unknown. Here we report the results of a longitudinal study of the reproductive performance of a genetically modified $B2m$ deficient mouse strain with low MHC Class I expression. Our data show that this mouse strain has an impaired reproductive performance. However, the mice superovulate well and show a normal estrous cycle. Breeding studies from crosses between the transgenic mice and the wild-type parental strain show that $B2m$ deficient mice have a significantly lower frequency of mating than the control $B2m^{+/+}$ mice. In addition, the litter size and weaning success of $B2m$ deficient mice were lower than the control. Perinatal lethality of the $B2m$ deficient offspring was also inflicted by cannibalism of the young pups by the $B2m$ deficient female. The impaired breeding phenotype (IBP) can be reversed by reintroducing the $B2m$ gene in F1 heterozygous $B2m^{+/-}$ animals; thus the presence of B2M confers a normal breeding pattern. The acquisition of an impaired breeding phenotype (IBP) as a result of the knockout of $B2m$ directly implicates B2M in the reproductive cycle of mice and raises the possibility of an effect of B2M on the reproduction of other mammals.

beta-2 microglobulin, female reproductive tract, fetus, MHC (H-2), odor discrimination, odortypes

INTRODUCTION

Beta-2 microglobulin (B2M) is the product of a single copy, low-polymorphic gene, which encodes a 12 000 Dalton nonglycosylated protein belonging to the immunoglobulin superfamily. The mouse $B2m$ gene is di-allelic, encoding two proteins that differ at a single amino acid residue [1]. B2M exists as a soluble product in serum, and, since its gene lacks a transmembrane coding exon, it is released into solution from a variety of cells. The B2M protein forms physical associations with a number of molecules; these include major histocompatibility complex (MHC) Class I and cluster of differentiation 1 (CD1) [2]. B2M also associates with the Fc receptor of the placenta, where it allows trans-placental transport of IgG from the mother to the foetus [3], and associates with the Fc receptor of neonatal gut epithelium for transport of IgG in the gut epithelium [4]. The major histocompatibility complex-related Fc receptor (FCGRT) is also implicated in IgG homeostasis in the adult [5]. In addition, it participates in the regulation of albumin [6] and in HFE (iron transport) [7].

The B2M protein plays a pivotal role in the biosynthesis of MHC class I molecules, controlling assembly, peptide binding, and cell-surface expression [8]. The majority of the fully assembled mature MHC class I molecules exist as an assembled complex with B2M, with the exception of free Class I heavy chains of the H-2^b haplotype [9]. Thus B2M is an essential molecule in the process of antigen presentation in the adaptive immune system.

One dramatic effect of the deletion of $B2m$ in homozygous knockout mice is that cell-surface and cytoplasmic expression of MHC Class I molecules are significantly reduced [10], leading to a reduction of the $CD4^-$ $CD8^+$ subpopulation of mature T cells [11, 12]. As a consequence, adult mice are susceptible to some parasitic diseases such as *Trypanosoma cruzi* infections [13], but not leishmaniasis [14] or blood-stage malarial infections [15]. These mice also have delayed viral clearance and increased mortality after Theiler virus infection [16]; and are susceptible to mouse hepatitis virus with the infected mice being more susceptible to acute hepatitis and encephalitis [17]. In rheovirus infection, $B2m^{-/-}$ mice showed enhanced intestinal mucosal and systemic immune responses by elevated IgG and IgA [18]. However, quite remarkably, in the absence of infections, a $B2m^{-/-}$ strain has a healthy phenotypic appearance, develops well into adulthood and has a normal life span, despite a deficiency in iron metabolism [19].

TABLE 1. Reproductive fitness of the *B2m* deficient mouse.

Mating combination	No. of litters born[a]	No. of pups born[a]
$B2m^{+/+}$ female $\times$ $B2m^{+/+}$ male	43 ± 2	315 ± 10
$B2m^{-/-}$ female $\times$ $B2m^{-/-}$ male	31 ± 4*	147 ± 7***
$B2m^{-/-}$ female $\times$ $B2m^{+/+}$ male	34 ± 6	134 ± 6***
$B2m^{+/+}$ female $\times$ $B2m^{-/-}$ male	35 ± 7	124 ± 9***
$B2m^{-/+}$ female $\times$ $B2m^{-/+}$ male	39 ± 4	400 ± 19*

[a] Values are presented as mean ± SEM.
* $P < 0.05$ and ***$P < 0.001$ versus wild-type females analyzed by Student *t*-test.

Of critical importance is the finding that *B2m* knockout mice are distinguishable by scent from identical mice with an intact *B2m* gene [20], thus supporting the notion that MHC products are important in providing olfactory recognition and odortypes in rodents [21–43] and humans [44–49].

In the course of experiments designed to generate mouse embryos for gene transcription studies and immuno-detection of MHC products [50–51], we consistently observed an impaired reproductive capacity in the *B2m* deficient mice. We report this observation here, which we have termed impaired breeding phenotype (IBP), and discuss the biological implications of such a defect.

MATERIALS AND METHODS

Mouse Strains and Crosses

All the mice in this study were of the C57BL/6 genetic background. Our transgenic founding colony consisted of females and males, all homozygous $B2m^{-/-}$ on the C57BL/6 background [11]. Mice from this colony, the wild-type parental strain ($B2m^{+/+}$ C57BL/6) and heterozygous offspring of crosses between these two strains were used. The frequency of mating was defined by the appearance of postcopulatory vaginal plugs after overnight pairing of single $B2m^{-/-}$, $B2m^{+/+}$, or $B2m^{-/+}$ males with groups of up to three females of $B2m^{-/-}$, $B2m^{-/+}$, or wild-type $B2m^{+/+}$ genotypes. Breeding data were collected over a period of 1 1/2 years from age-matched crosses generated between $B2m^{-/-} \times B2m^{-/-}$ (20 pairs of mice; breeding period: 6–32 weeks of age); $B2m^{+/+} \times B2m^{+/+}$ (10 pairs of mice; breeding period: 6–32 weeks); $B2m^{+/+} \times B2m^{-/-}$ (8 pairs of mice; breeding period: 6–24 weeks); $B2m^{-/-} \times B2m^{+/+}$ (10 pairs of mice; breeding period: 6–28 weeks) and $B2m^{+/-} \times B2m^{+/-}$ (33 pairs of mice; breeding period: 6–24 weeks). Once a mouse was seen to be pregnant, the male was removed from the breeding pair and the female was housed alone until her pups were weaned. Thus stress due to overcrowding or the presence of the male was avoided. All procedures described within were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Essex and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Mouse Husbandry

In order to avoid shifts in the circadian rhythm and hence the estrous cycle, all mice were bred and maintained in a temperature-, humidity-, and light-controlled room (lights-on 700 to 2000 h) and housed in identical cages (University of Essex, Biological Services Unit). Mice were allowed food (laboratory diet RM3 [E], SDS Ltd., Witham, Essex) and water ad libitum. Our mouse colony was free of mouse hepatitis virus.

Cannibalism

This behavioral characteristic was defined as a defect in the normal care of the newborn pups, reflected in visible physical damage to the pups and detachment of the mother from the newborn litter.

Superovulation Procedure

Adult (6–8 weeks of age) females were stimulated with 5 IU eCG (Folligon, Intervet UK Ltd., Milton Keynes, UK) and 5 IU hCG intraperitoneal (Pregnyl, Organon Laboratories Ltd., Cambridge, UK) at 1700 h, 48 h apart, to induce
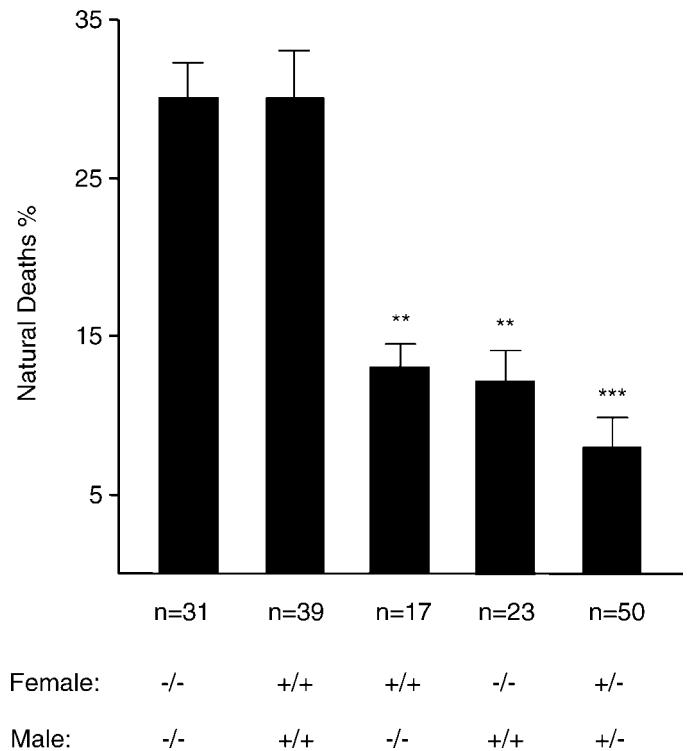

FIG. 1. Percentage of natural death is normal in $B2m^{-/-}$ deficient mice. Percentage of natural deaths occurring before weaning is indicated for $B2m^{-/-}$ deficient (19.9%), $B2m^{+/+}$ wild-type (29.8%), $B2m^{+/+} \times B2m^{-/-}$ (12.9%), $B2m^{-/-} \times B2m^{+/+}$ (11.8%), and $B2m^{+/-} \times B2m^{+/-}$ (6.8%). Number of mating crosses (N) is shown. Percentage of natural deaths occurring with $B2m^{-/-}$ genotype was comparable to the $B2m^{+/+}$ wild-type C57BL/6. Percentage natural deaths fell for the $B2m^{+/+} \times B2m^{-/-}$ and the $B2m^{+/-} \times B2m^{+/-}$ mating. Results are expressed as mean ± SEM. Chi-square test was used to determine differences between the groups. **$P \leq 0.01$; and ***$P \leq 0.001$.

superovulation. The control group consisted of naturally cycling females of the same strain (without stimulation).

Determination of Estrous Cycle

The estrous cycle was studied using cells removed by gentle mechanical disruption following vaginal washes, stained with hematoxylin/eosin solution and analyzed by light microscopy. Samples were classified as follows: proestrus, nucleated epithelial cells with occasional leukocytes and small degenerative nuclei cells; estrus, numerous cells from squamous epithelium and small epithelial cells; metestrus, numerous leukocytes and small cornified epithelial cells, and diestrus, mucus and nucleated cells.

Data Analysis

Data are expressed as means and SEM. Statistical significance between groups was tested using chi-square test for homogeneity and Mann-Whitney *U*-test for two proportions. Significance was set at $P \leq 0.05$ (*), $P \leq 0.01$(**), $P \leq 0.001$(***).

RESULTS

Reproductive Fitness

A longitudinal study designed to quantify the reproductive fitness of the *B2m* deficient mouse was conducted. Due to a steady depletion of breeding stocks of the transgenic mice, we began to outbreed the transgenic *B2m* deficient C57BL/6 mice with wild-type C57BL/6 mice to produce *B2m* heterozygous stocks. The F1 litters from these mating were then crossed to
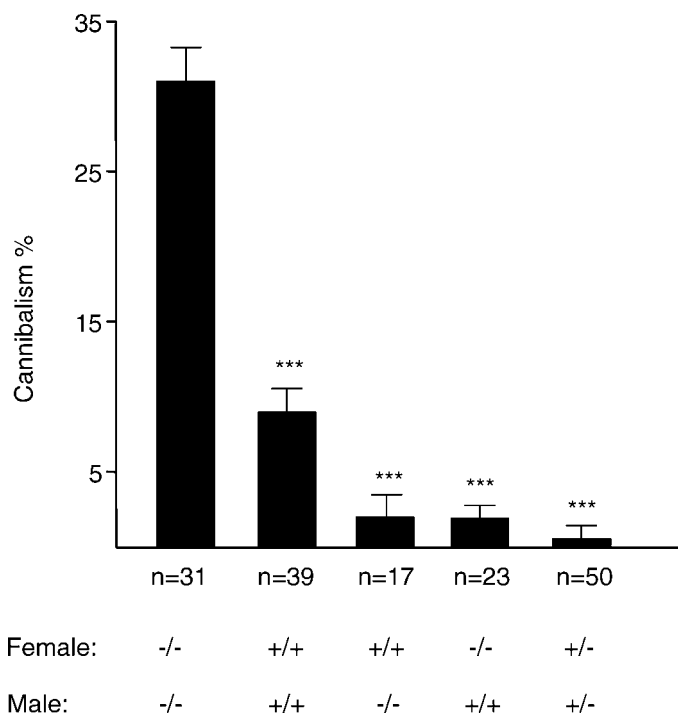
FIG. 2. Cannibalistic behavior by the mother is increased on a $B2m^{-/-}$ background. Percentage of pups cannibalized by the mother is indicated for all four mating crosses $B2m^{-/-} \times B2m^{-/-}$ (31.3%), $B2m^{+/+} \times B2m^{+/+}$ (9.2%), $B2m^{+/+} \times B2m^{-/-}$ (2.4%) $B2m^{-/-} \times B2m^{+/+}$ (2.1%) and $B2m^{+/-} \times B2m^{+/-}$ (0.5%). Number of mating crosses (N) is indicated. Incidence of cannibalism falls as $B2m$ is reintroduced into the genotype of the mice. Results are expressed as mean $\pm$ SEM. Chi-square test was used to determine differences between the groups. ***$P \leq 0.001$.

FIG. 3. Weaning success is increased on a $B2m$-positive background. Weaned percentage is illustrated for all four mating crosses; $B2m^{-/-} \times B2m^{-/-}$ (38.8%), $B2m^{+/+} \times B2m^{+/+}$ (60.9%), $B2m^{+/+} \times B2m^{-/-}$ (84.7%), $B2m^{-/-} \times B2m^{+/+}$ (87.5%) and $B2m^{+/-} \times B2m^{+/-}$ (92.8%). Number of mating crosses (N) is indicated. Due to a high degree of cannibalism in the $B2m^{-/-}$-deficient mice, the weaned success is lower compared with the $B2m^{+/+}$ wild-type mice. Incidence of natural deaths and cannibalism falls for the $B2m^{+/+} \times B2m^{-/-}$ and $B2m \times B2m^{+/-}$ mating crosses, and hence weaning success increases. Results are expressed as mean $\pm$ SEM. Chi-square test was used to determine differences between the groups. ***$P \leq 0.001$.

replenish the $B2m$ negative mice. The data show that the $B2m$ deficient mice have a lower number of litters born than the control group of C57BL/6 mice ($P < 0.05$). The total number of pups born to $B2m$ deficient mice was significantly lower than the number born to the control mice ($P < 0.001$) (Table 1).

Postnatal Mortality

Analysis of postnatal mortality as determined by natural death (not cannibalized animals) (Fig. 1), revealed that the $B2m$ homozygous deficient mice ($B2m^{-/-}$) had a similar proportion of postnatal natural deaths (29.9%) to the C57BL/6 $B2m^{+/+}$ homozygous control mice (29.8%), although the control group had a higher total number of pups born. Interestingly, overall postnatal natural death declined when a $B2m^{+/+}$ female was crossed with a $B2m^{-/-}$ male (12.9%) or a $B2m^{-/-}$ female was crossed with a $B2m^{+/+}$ male (11.8%) ($P < 0.01$). This decline was even greater when the heterozygous $B2m^{+/-}$ mouse strain was crossed with identical $B2m^{+/-}$ mice (6.8%) ($P < 0.001$), suggesting that crossing mice heterozygous for $B2m$ confers a higher rate of survival of the offspring. Since the progeny of this cross will include genotypes of $B2m^{+/+}$, $B2m^{+/-}$, or $B2m^{-/-}$, this finding suggests that the maternal genotype may be an important determining factor rather than the embryo genotype.

Cannibalism

We then examined the postnatal mortality rate by cannibalism (Fig. 2) and observed that pups born from homozygous $B2m^{-/-}$ parents exhibited a significantly higher rate of death than pups born to the $B2m^{+/+}$ mice (31.3% and
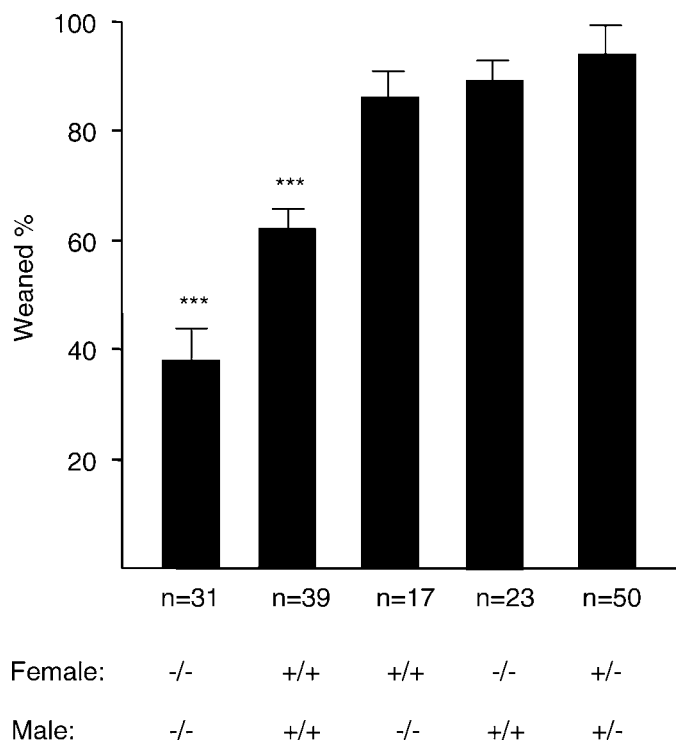
9.2% of total pups born respectively [$P < 0.001$]). The presence of the $B2m$ gene appears to confer a higher survival rate since the homozygous $B2m^{+/+}$ female mice crossed with the homozygous $B2m^{-/-}$ males exhibited a low cannibalism death rate (2.4%). The reverse mating combination that is homozygous $B2m^{-/-}$ female mice crossed with the homozygous $B2m^{+/+}$ males also exhibited a low cannibalism death rate (2.1%). Similarly, the mating of $B2m^{+/-}$ heterozygous individuals showed a lower death rate by cannibalism (0.5% [$P < 0.001$]).

Weaning Success

The impact of the aforementioned variables on the weaning success of the $B2m^{-/-}$ deficient mice as measured by the survival rate of the pups to adulthood was examined (Fig. 3). A measure of association between the survival rate and the mating genotypes revealed a high statistical significance ($P < 0.001$). Mice born to a cross between heterozygous $B2m^{+/-}$ females and $B2m^{+/-}$ males exhibited the highest survival rate. Similar levels of significance were observed between the rate of mortality (natural death plus cannibalism) and the type of mating ($P < 0.001$). Pups born to $B2m^{-/-}$ parents showed the poorest survival rate. In addition, the $B2m^{-/-}$ mice showed a very significant disproportionate increase in postnatal mortality due to cannibalism when compared with the mice carrying the $B2m$ gene ($P < 0.001$).

TABLE 2. Number of ovulating mice and number of ovulated oocytes per animal from stimulated adult females and naturally cycling control females.

Female	Treatment	No. of mice that ovulated	No. of ovulated oocytes (range)[a]
Natural controls	None	10/10	18.0 ± 0.8 (15–20)
$B2m^{+/+}$	5 IU (hCG) + 5 IU (eCG)	10/10	59.0 ± 4.9 (48–77)**
$B2m^{+/-}$	5 IU (hCG) + 5 IU (eCG)	10/10	63.2 ± 4.8 (52–80)**
$B2m^{-/-}$	5 IU (hCG) + 5 IU (eCG)	10/10	61.7 ± 3.7 (50–78)**

[a] Values are presented as mean ± SEM (n = 10).
** $P < 0.01$ versus control group analyzed by Student t-test.

Mating Frequency, Superovulation, and Estrous Cycle

Mating frequency was significantly reduced in the $B2m$ deficient dams compared with the $B2m^{+/+}$ wild-type mice. A reduction of the percentage of mating on Night 1 to Night 4 on $B2m$ deficient (66 ± 3, $P < 0.01$) versus the $B2m$ wild-type mice (90 ± 5) was observed. I. nterestingly, the capacity of the $B2m$-deficient mouse strain to respond to superovulation with the hormone hCG and eCG was similar to other mouse strains (Table 2). Moreover, there was no difference in the estrous cycle analysis between $B2m$ deficient and wild-type female mice (Fig. 4). These results suggest that the $B2m^{-/-}$ mice have an intact and responding corpus luteum and therefore a normal ovulation pattern. The possibility of resorption of the entire litter as a cause of poor breeding was ruled out, since mating of a $B2m^{-/-}$ female, detected by the presence of vaginal plugs, normally led to a successful pregnancy and the birth of a litter.

DISCUSSION

In this study we have shown that the $B2m$ knockout mouse has an impaired breeding phenotype (IBP). The results implicate the absence of B2M, and consequently diminished expression of MHC Class I in the altered reproductive performance of this mouse strain. The female $B2m^{-/-}$ mice also exhibited a high level of cannibalism towards the newborn pups, suggestive of an alteration of the maternal nurturing behavior and the mechanism that control progeny recognition by the mother. The cannibalistic behavior of the $B2m^{-/-}$ mice extends observations on the relationship of offspring recognition by the mother, and communal nesting and communal nursing in mice [19].

B2M is found in the serum and body fluids, both in a free form and associated with MHC class I heavy chain. Previous studies have shown that soluble MHC Class I molecules can act as pheromones, influencing mating signals within rodent colonies [35–39]. Therefore, a possible mechanism operating in the $B2m$-deficient mice is the absence of secreted MHC class I products, and thus the absence of mating signals resulting in impaired breeding. This mechanism might also render the female unable to recognize the offspring as her own, leading to cannibalistic behavior. It has been postulated that MHC genes act as kin-recognition markers to increase relatedness among communal nesting partners [21, 24, 52]. Mice can recognize one another by individual characteristic body odors that reflect their genetic constitution at the MHC [22] and mothers are able to recognize syngeneic pups from other pups differing only in
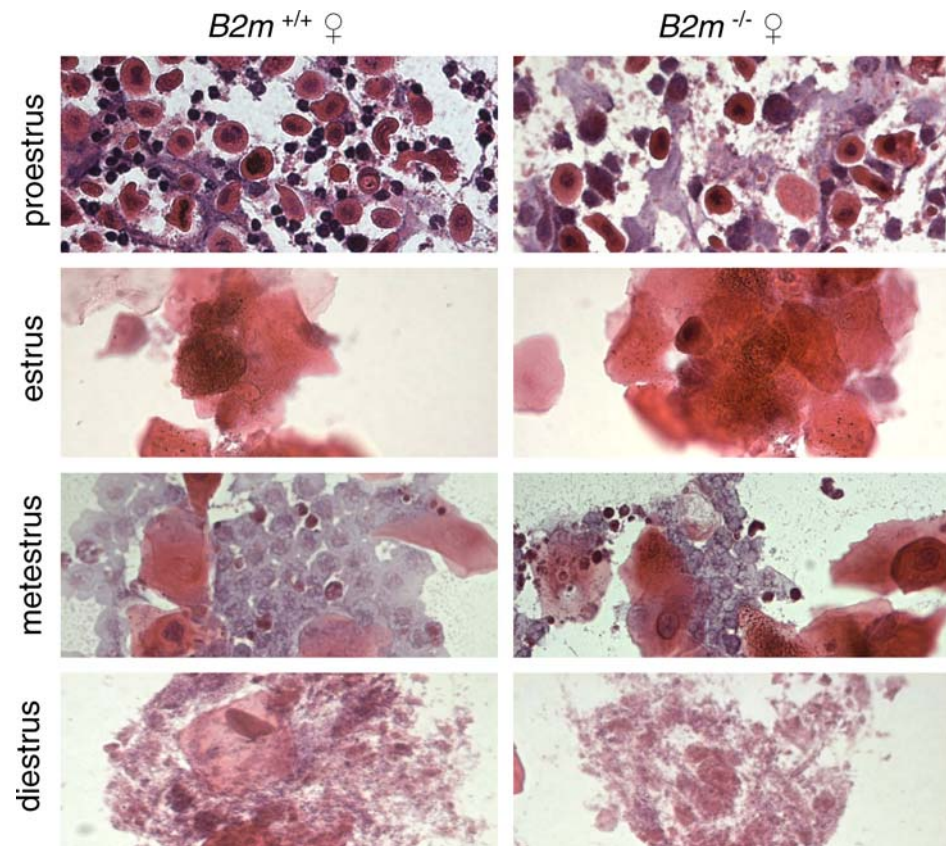

FIG. 4. Estrous cycle in vaginal fluid samples from $B2m$-deficient and wild-type female mice. Representative images of the samples from proestrus, estrus, metestrus, and diestrus (estrous cycle) stained with hematoxylin/eosin. Original magnification ×400.

MHC [23]. Pup lethality, a form of cannibalism affecting female nurturing behavior, has also been reported as a result of *fosB* gene deficiency [53]; although in a different context, the phenotypic effect bears some similarity to the effect of the *B2m* deficiency. In humans, there is also data suggesting that MHC genes are linked to olfactory receptors and act as odor recognition cues in mating choices [44–49].

The homozygous *B2m*$^{-/-}$ and heterozygous *B2m*$^{+/-}$ females produce the same number of oocytes following superovulation as the control *B2m*$^{+/+}$ females, suggesting that they should produce as many offspring per mating as their wild-type counterparts. However, litter sizes are smaller indicating that a lack of *B2m* impairs embryo development. The *B2m*$^{-/-}$ mice have a severely diminished expression of MHC Class I products both in the adult tissues and throughout development. A reasonable postulate is that B2M and, in turn, MHC deficiency are implicated in the poor breeding capacity of the *B2m* deficient mice by affecting mating, embryo development, and maternal behavior.

The IBP phenomenon can be rescued by introducing the *B2m* gene back into the genome of the mice. Breeding experiments in which C57BL/6 *B2m*$^{-/-}$ transgenic mice were crossed with the C57BL/6 *B2m*$^{+/+}$ parental mouse strain restored the reproductive pattern of the heterozygous progeny to normality. These observations are consistent with the notion that MHC disassortative mating preferences, driven probably by pathogens, favor MHC heterozygosity [54].

The impact of variations in the maternal uterine genotype and maternal cytoplasmic contributions to the oocyte has been controlled by use of mice of identical C57BL/6 genetic background in all crosses. The only genetic variable is the presence or absence of functional *B2m* allele(s). In previous studies, we have shown that C57BL/6 (H2-D^b) derived MHC Class I products are synthesized at the one-cell embryo stage, arguing for a requirement of MHC in development [55]. This finding was confirmed in another mouse strain; Arcellana-Panlilio and Schultsz showed that in the CD-1 Swiss albino mice, H-2 K^q products were also expressed at all stages of preimplantation development [56]. The exact mechanism for a role of MHC in early development is still unclear. It may involve stage-specific antigen synthesis to allow differentiation and cell division, similar to the role of differentiation molecules involved in the ontogeny of lymphocytes [57]. MHC-mediated transmembrane signaling pathways, particularly in the case of H2-Q products, which have glycosidylinositolphosphate, anchored transmembrane domains [58] might also be a signal mechanism operating in mammalian embryogenesis. We have postulated that a background level of MHC Class I is required for successful mouse development and for protection against maternal large granular cells and natural killer cells (NK) [59], as it has become established that in the adult, NK effector cell recognition involves targeting cells with diminished MHC expression [60].

Conversely, it has also been shown that overexpression of MHC Class I products has a detrimental effect on development. Indeed, Jaffe and colleagues have shown that embryos overexpressing H-2D^d antigen under the control of the human beta-actin promoter, when introduced into pseudopregnant syngeneic foster females fail to develop past the mid-point of gestation; while control embryos that overexpressed an irrelevant protein developed normally [61]. The above observations suggest that a homeostatic level of MHC might be protective and influence normal development and viability early in life. It is possible that in the *B2m*$^{-/-}$ mouse strain the lack of B2M impairs the synthesis and cell-surface expression of MHC Class I, thus altering the normal balance of MHC

expression during early stages of development. B2M normally associates with the Fc receptor of the placenta [3], therefore, in the pregnant female *B2m*$^{-/-}$ mice, the Fc receptor may have a reduced capacity to transport IgG molecules across the placenta to the developing embryo, affecting development and reducing litter size.

Taken together these results indicate that the *B2m* deficient mouse strain has an impaired reproductive pattern, that we have termed Impaired Breeding Phenotype (IBP). The acquisition of IBP as a result of selective genetic deficiency of *B2m* directly implicates the B2M protein in the reproductive cycle of mice and raises the possibility of an effect of B2M in the reproduction in humans.

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