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Microphthalmic Mice Display a B Cell Deficiency Similar to that Seen for Mast and NK Cells

Kirstin Roundy, Angela Kollhoff, E. J. Eichwald, Janis J. Weis, and John H. Weis

The microphthalmic mouse (mi) possesses a 3-bp deletion of the Mi gene that alters the DNA binding site of the transcription factor gene product. This animal has diminished numbers of NK and mast cells (MC) and is osteopetrotic due to a lack of the normal complement of functional osteoclasts. The reduction of MC has been proposed to be due to the lack of adequate c-Kit expression that is required for MC differentiation. However, data from other labs has questioned this interpretation. In this report, we present data suggesting bone marrow-derived deficiencies of the mi mouse are not due to a lack of c-Kit expression and function, but instead due to an inhospitable environment within the bone marrow itself. Specifically, we have found that such animals also lack virtually all B cell precursors within the marrow and rely upon other lymphatic sites, such as the spleen, for B cell development and maturation. Although the animal has depressed numbers of NK cells, B cells, and MC, it still possesses a normal thymus and peripheral T cells. Therefore, the block in cellular differentiation must be within the marrow environment, which is essential for maturing B cells, NK cells, and MC but not T cells. The Journal of Immunology, 1999, 163: 6671–6678.

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3 Abbreviations used in this paper: bHLH-Zip, basic helix-loop-helix-leucine zipper; MC, mast cell; mi, microphthalmia; SCF, stem cell factor; MMCP, mastocytosis MC protease; OPGL, osteoprotegrin ligand.

T
he microphthalmia locus encodes a basic helix-loop-helix-leucine zipper (bHLH-Zip) transcription factor (Mi) (1, 2) that is closely related to, and heterodimerizes with, a family of at least three other transcription factors (TFE3, TFEB, and TFE3C) (3–7). There are a variety of mutations of the Mi gene that give rise to a series of phenotypes including small eyes and reduced eye pigmentation, deafness, lack of fur pigmentation, osteoporosis, and reduced numbers of mast cells (MC) and NK cells (for review see Ref. 8). The most commonly studied mutation of the microphthalmia gene is mi, which possesses a 3-bp deletion in that region of the sequence that dictates site-specific DNA binding by the mature protein (1, 2). Thus, the mi mutation ablates DNA binding by both mi/mi homodimers and heterodimers of mi and the other members of the protein family (9). This dominant-negative mutation is evidenced by partial phenotype in the heterozygote. Similar mutations in the human gene give rise to Waardenburg syndrome, which is characterized by hearing loss and pigmentation anomalies (10).

Specific DNA sequences are recognized by the basic region of the bHLH-Zip protein (for review see Ref. 11). The core sequence recognized by virtually all members of the bHLH-Zip family is a conserved E box sequence, CANNTG. Mi, as a member of this family, recognizes a subset of E box sequences, notably those of the M box sequence CATGTG (12). The basic region of Mi, either alone as a homodimer or as a heterodimer with TFE3, TFEB, and TFE3C, recognizes the M box sequence (13). The genes tyrosinase and tyrosinase-related protein 1 (TRP-1), which are not expressed by animals of the mi/mi genotype, possess this M box sequence (12, 14).

The effect of Mi on the development of bone marrow-derived cells has primarily focused upon deficiencies in the numbers of, and function of, MC, NK, basophils, osteoclasts, and macrophages in animals with the +/mi and mi/mi genotype. Early work documented the reduced numbers of MC, NK cells, and basophils in mi (mi/mi) and heterozygote (+/mi) animals compared with the wild type (+/+ ) (15, 16). For example, the +/+ animal possessed ~3-fold more peritoneal MC and 10-fold more dermal MC than the mi/mi animal, while the +/mi animal possesses intermediate numbers. These data resulted in the proposal that the intrinsic defect giving rise to the reduced numbers of such cells was phenotypically active within the cells themselves and that defective bone marrow was not responsible for such reductions.

The paucity of MC in the mi/mi and +/mi animals has been proposed to be due to inadequate cell-surface expression of the c-Kit tyrosine kinase receptor to allow for MC differentiation and expansion (17). FACS analysis of spleen-derived cultured MC demonstrated virtually no c-Kit expression on the surface of the mi/mi cells compared with the analogous cells from the +/+ animals, which displayed nearly a 2 log increase of c-Kit staining over the negative Ab control. Extension of this investigation by sequence and reporter gene analysis suggested that the c-Kit gene possessed sequences that were under the transcriptional control of the Mi gene product (18). However, the M box sequences demonstrated to be critical for recognition by Mi protein were not those of the typical E box element, but instead an atypical sequence of CACGTGCCAGGTG. MC derived from mi animals lack expression of mouse MC protease 6 (MMCP-6), the gene of which contains the canonical M box sequence (19, 20).

An alternative model for MC deficiencies in animals possessing the mi mutation was suggested in complementation assays with the c-fms gene product (21). The c-Kit and c-fms receptor tyrosine kinases are closely related and have as ligands stem cell factor (SCF; also known as MC growth factor, MGF; steel ligand, S) and CSF-1, respectively. MC derived from animals lacking the c-Kit
protein (mutations within the W locus) die when placed on a fibroblast monolayer (a source of SCF and CSF-1). These cells survive in such a coculture when provided with an expression construct of c-fms. These data suggested that both c-fms and c-Kit activate MC in the same or overlapping pathway. However, when cultured MC from mi/mi animals were placed within such an assay, they could not be rescued by c-fms expression, suggesting their cellular defect is downstream of c-Kit expression. This model was extended by a recent finding that binding of c-Kit with SCF induces melanocyte mitogen-activated protein kinase-dependent phosphorylation of the Mi protein at a consensus serine (22). This phosphorylation event resulted in an up-regulation of Mi-dependent transcription of the tyrosinase pigment gene. Thus, c-Kit activation directly activated Mi protein function.

The apparent contradictions between these two models became more obvious when Mi-dependent transcriptional activation of the c-Kit gene was re-examined due to further analysis of E box binding sites for the Mi protein (13). In this study, the E box elements of the c-Kit promoter were analyzed for Mi binding via EMSA and yeast one hybrid analysis; the atypical E box of the c-Kit gene did not demonstrate significant Mi binding and did not serve to activate a yeast one hybrid system via Mi binding. However, the c-Kit E box was effective at using MyoD for such activation, suggesting that this sequence is not recognized by Mi but by another member(s) of the bHLH-Zip protein family.

It was with these discrepancies in mind that we initiated a reanalysis of MC growth and function in the mi mouse. We have found that the expression of c-Kit protein on the surface of cultured MC from the mi/mi animal is at virtually the same level as that of the +/mi and +/- mi animals. Colony-forming assays demonstrated that the bone marrow of mi/mi animals was deficient in CFU for IL-3-, SCF-, or IL-7-dependent outgrowth. FACS analysis of mi/mi marrow confirmed the absence of CD19-, B220-, and IL-7R-bearing cells, suggesting a lack of precursor B cells. Reduced numbers of normal, mature B cells could be found in the periphery, similar to the reductions seen in peripheral MC numbers. These data suggest that the primary defect in mast cell and B cell numbers in the mi/mi mouse (and by inference the reduced numbers of NK cells and basophils) is primarily due to the environment of the marrow itself and not due solely to decreased cellular function of the various bone marrow lineages.

Materials and Methods

Animals, cell growth, and analysis

Animals. A heterozygote microphthalmia breeding pair (mi mutation) was obtained from The Jackson Laboratory (Bar Harbor, ME). A breeding colony was established and maintained under pathogen-free conditions. +/- mi, +/+ mi, and mi/mi littersmates were sacrificed by cervical dislocation at 3–7 wk of age.

Cell preparation. Mouse IL-3-dependent bone marrow-derived mastocytoma CT26 were maintained as previously described (23). Maturing connective tissue-like MC were cultured in SCF as previously described (24). Maturing MC were derived in WEHI supernatant as previously described (25). All adherent cells were removed from these cultures before any analysis of the maturing MC was performed.

Single-cell suspensions were prepared from spleen, bone marrow, and Peyer’s patches of sacrificed animals. Spleens were removed and mechanically disrupted by passage through 0.2-μm nylon cell strainers. Mouse femurs and tibias of +/- mi and +/+ mi mice were flushed with media (RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS (HyClone, Logan, UT) and 1% Pen-strep (Life Technologies)). Marrow from mi/mi mice was obtained by finely dissecting the bone to release the cells. Spleen and bone marrow cell suspensions were then centrifuged and re-suspended in 2 ml ACK lysis buffer (0.15 M NH₄ Cl, 1.0 M KHCO₃, 0.1 mM Na₂EDTA) for 10 min to lyse RBC. Cells were centrifuged and re-suspended in 1× PBS, 0.1% BSA for cell staining or RPMI 1640 for use in the colony-forming assay. Peyer’s patches were removed from in vivo in the colony-forming assay. Peyer’s patches were removed from the various bone marrow lineages.

Results

Expression of the c-Kit receptor by spleen- and bone marrow-derived MC

The depressed numbers of MC within mi/mi and +/mi mice has been suggested to be due to the low level of expression of the c-Kit tyrosine kinase (17). Studies with mice genetically deficient for a functional c-Kit protein have demonstrated the obligate role of this receptor for MC development (28). Previous analyses for c-Kit expression by FACS were done using MC derived from splenic cells cultured in WEHI-3 supernatant (17). We prepared analogous cells from the spleen and bone marrow of wild-type (+/+), heterozygous (+/mi), and mi (mi/mi) animals using either IL-3 and/or SCF for MC expansion (29). Such cells possessed the usual MC granule staining morphology and expressed a number of MC-specific/enriched products (see below). Although, initially, there were fewer MC within the mi/mi and +/- mi cultures (compared with
+/-), the cell numbers quickly expanded to virtually the same end point (data not shown). FACS analysis of splenic-derived MC (Fig. 1A) and bone marrow-derived MC (Fig. 1B) using a c-Kit-specific mAb demonstrated a high level of c-Kit on the surface of such cells regardless of their genetic derivation or the growth factor used to derive the cells. These results are in contrast to a previous publication that demonstrated negligible c-Kit expression on cultured MC from mi/mi mice (17).

**Transcript analysis of ear tissue and cultured MC**

To evaluate MC products in vivo, we isolated total RNA from the ears of 10-day-old mice and SCF-derived MC and analyzed them for a variety of transcripts. We used a semiquantitative RT-PCR protocol optimized for very fast cycle times. As a control, we developed an oligonucleotide set that discriminates between the larger, normal form of the Mi protein compared with the shorter, mutant mi form. By resolving these two products in a denaturing sequencing gel (Fig. 2), it was possible to display the difference in the Mi gene products from the +/-, +/-, and mi/mi animals. As shown in Fig. 2 (left), RNA derived from the 10-day-old ear possessed approximately equivalent transcript levels for c-Kit and beta-actin between the three genotypes. MMCP-6, which is not transcribed in mi mast cells (19, 30), was totally diminished in the mi/mi sample as were transcripts for the tyrosinase gene, which is not transcribed in mi melanocytes. The absence of MMCP-6 transcripts and presence of c-Kit transcripts in the mi/mi tissue suggests these two genes are not controlled by the same transcriptional apparatus.

Transcript analysis of splenic MC derived in SCF from wild-type (+/-) and mi (mi/mi) animals (Fig. 2, right) demonstrated that the expression of several MC products such as TNF and the gamma-chain of the FceR1 IgE receptor are unchanged in the mutant cells. There is little if any diminution in c-Kit transcripts in the mi/mi MC. These data are representative of a number of similar experiments performed on spleen- and bone marrow-derived MC (via IL-3 or SCF differentiation for a variety of weeks) in which such cells lack appreciable transcripts for MMCP-6 (data not shown) but possess normal, or only slightly reduced, levels for other MC products.

**Growth characteristics of mi/mi mast cells**

Mast cells derived from mi/mi animals are presumed to be at a growth disadvantage compared with the wild-type cells due to a depressed number of c-Kit receptors. Alternatively, the reduced numbers of MC within the mature animals could be due to suppressed numbers of precursor cells, which would in turn give rise to fewer numbers of mature cells. One assay to determine the relative fitness of derived cell lineages is to compare their division times under optimal growth conditions (Fig. 3). MC were derived from splenic precursors in the presence of WEHI supernatant, SCF or IL-3, and analyzed for [3H]thymidine uptake using two different cell concentrations. MC derived in WEHI and IL-3 grew at similar rates whether they were obtained from the mi/mi mouse or wild

**FIGURE 2.** Gene expression in the ears of 10-day-old mice and SCF-derived MC. Total RNA was obtained from the ears of 10-day-old mice of the genotype noted and from SCF-derived MC (30 days in culture) and analyzed by RT-PCR using oligonucleotides specific for the genes noted. *, Wild-type Mi allele sequence; #, mi allele that is 3-bp smaller than wild type. The genetic source of the samples was denoted as +/- (wild type), +/mi (homozygous for the mi allele), and mi/mi (homozygous for the mi allele). PCR cycle numbers: 10-day ear: beta-actin, 15 cycles; MMCP-6, 23 cycles; c-Kit, 21 cycles; tyrosinase and Mi/mi, 26 cycles; SCF-MC: beta-actin, 15 cycles; c-Kit, 19 cycles; TNF, 23 cycles; IgE, 17 cycles; Mi/mi, 28 cycles.

**FIGURE 1.** Detection of c-Kit on the surface of spleen- and bone marrow-derived MC obtained from mi and wild-type mice. MC were grown from splenocytes (A) or bone marrow samples (B) for varying lengths of time in culture (denoted as 20 day, etc.) with either SCF and/or IL-3. Cells were stained for c-Kit expression (dark peak) with the appropriate isotype control (light peak). The genetic source of the samples was denoted as +/- (wild type), +/- (heterozygotes), and mi/mi (homozygous for the mi allele).
type. However, the mi/mi MC maintained in SCF did show a diminished growth rate compared with the normal cells. That these cells could be derived and expanded in media in which SCF was the only growth factor indicates c-Kit activation must influence more than just the Mi activation pathway. It also suggests that the Mi gene product does influence the expression of other gene products that, in a SCF-only culture, diminish the fitness of the cell. In addition, the IL-3 differentiation pathway must be independent of Mi because there is no significant difference in the growth kinetics of the mutant or wild-type cells in an IL-3-only culture media.

**FIGURE 3.** Growth rates of mi mast cells in various cytokines. Splenocyte MC were derived from wild-type (+/+ ) or mi animals (mi/mi) using either WEHI supernatant, SCF, or IL-3 for a period of at least 25 days. Cells were washed and incubated at varying cell numbers for 4 h in the presence of [3H]thymidine and fresh cytokine. The cells were isolated, washed, lysed, and counted in triplicate. The data presented is from one experiment but is representative of at least three independent analyses.

**Enumeration of CFU from spleen and bone marrow in the presence of SCF, IL-3, or IL-7**

Examination of cross sections of the osteopetrotic femur bone obtained from mi/mi mice demonstrates not only a major loss of marrow cavity, but suggests that the environment of the marrow has also been altered. The effect of this alteration can be tested, in part, by enumeration of precursor cells within the mi/mi marrow cavity via CFU analysis. Mouse bone marrow was isolated from mi/mi, +/+ , and +/+ animals by mechanical disruption of the bone. Cells were counted and grown in semisotil agar in the presence of SCF, IL-3, or IL-7. After 7 days in culture, CFU were counted. Wild-type and heterozygote animals gave virtually the same results (data not shown). CFU derived in IL-3 were enriched in +/+ marrow, and less so in spleen, while the bulk of CFU forming potential in the mi/mi mouse was within the spleen (Fig. 4). Alternatively, the majority of SCF-dependent CFU were still present within marrow cavity of both animals albeit the mi/mi marrow sample possessed one-third to one-half fewer precursor cells compared with the +/+ marrow. IL-7 is a lymphopoietin that is required for appropriate differentiation of T and B cells (31). The vast majority of IL-7 responsive cells in normal bone marrow are developing B cells. Interestingly, when marrow samples from mi/mi mice were analyzed, there were virtually no detectable IL-7-dependent CFUs generated (Fig. 4). This is in stark contrast to that of the heterozygote or wild-type animal (data not shown). These data suggest that the configuration of the mi/mi bone marrow is altered in such a way as to preferentially exclude precursor cells of particular hemopoietic potential.

**Absence of developing B cells in the mi/mi bone marrow**

The absence of IL-7 responsive CFU from mi/mi marrow samples suggested that B cell precursor cells were absent. Accordingly, marrow samples from mi/mi and +/+ animals were analyzed for cell-surface markers indicative of B cell lineage (Fig. 5). B220 is an isofrom of CD45 that is present on B cells and NK cells, while CD19 is only present on maturing/mature B cells. The mi/mi marrow possessed about 10 and 16% of cells that stained with CD19 and B220, respectively, compared with 42 and 45% of positive cells for these two markers with wild-type marrow. A similar change in positively staining cells, 10 and 37% for mi/mi and wild type, respectively, was seen when an Ab specific for the IL-7 receptor was used. The marrow of a normal animal can be broadly divided into those cells of B cell (CD19 positive) or granulocyte lineage (Gr-1 positive). Few, if any, cells are both CD19 and Gr-1 positive (data not shown). If the mi/mi marrow sample is deficient in maturing B cells, it should then be enriched for Gr-1-positive cells. As shown in Fig. 5, the majority of marrow cells from the mi/mi animals are Gr-1 positive (70%), while the wild-type marrow consists of about 40% Gr-1-positive cells.

Previous analyses of the number of mature MC and NK in the mi/mi mouse enumerated a diminution of such cells in the range of 10–25% (either by cell count or activity) of wild-type levels. Because the mi/mi bone marrow possessed only a very small percentage of B cell precursor cells, it was of interest to determine whether the animal possessed peripheral B cells and whether their numbers would be reduced to a similar percentage as that of the MC. Splenic samples were analyzed from mi/mi and wild-type animals for T and B cell populations using CD19, B220, and CD3 as cell-surface markers. As shown in Fig. 6, 35% of the cells from the mi/mi spleen stained with B220, 16% stained with CD19, and 41% of the cells expressed CD3. This is in contrast to the wild-type spleen, of which 60% of the cells expressed B220, 50% expressed CD19, and 21% were CD3-positive cells. The exact percentage
loss of B cells within the mi/mi spleen (and thus increase of CD3+ T cells) varies among different animals, yet all show a consistent loss compared with the wild type (data not shown).

Cellular constituents and morphology of mi/mi Peyer’s patches

The lack of maturing B cells in the mutant bone marrow and diminished numbers of B cells in the mi spleen suggested that either the mi mutation affects the B cells directly in a transcriptional cascade or that the mi marrow is not a conducive site for B cell development. A report by Kim et al. recently suggested that MC derived from mi/mi mice did not express appreciable levels of the α4 integrin chain (32) due to a CACTTG E box motif at position −294 in the promoter region. This E box is not an M box nor was this sequence demonstrated as significantly influencing α4 expression in a separate analysis (33). Mouse α4 knockout studies have noted that α4-deficient B cell precursors fail to develop in the marrow. Such mice had no detectable Peyer’s patches, and the B cells present within the spleen were immature and lacked appreciable expression of either B220 or surface IgM (34, 35).

The question these data raised was whether the absence of B cells in the mi marrow was due to lack of α4 expression by B cells or whether the bone marrow environment simply could not support the development of such cells, forcing peripheral lymphoid tissues to take on a lymphopoietic function. If the expression of α4 does require the mi gene product, then an analysis of tissues for α4 expression should show a loss of expression, similar to that seen for MMCP-6 in MC and tyrosinase in melanocytes. RT-PCR analysis of a variety of lymphoid tissues (spleen, thymus, intestine/ Peyer’s patches, and bone marrow) clearly demonstrated α4 expression in the mi/mi animal virtually identical with that of the +/mi animal (Fig. 7). The expression of α4 in the heterozygote is virtually the same as that seen for wild type (data not shown). The major difference seen between the mutant and heterozygote animal is obvious in the bone marrow, which lacks appreciable levels of α4 transcripts in the mi/mi sample. The absence of α4 transcripts in this sample is most likely due to the absence of B cell precursors within the mi/mi marrow. Thus, the mi mutation does not appreciably influence the expression of the α4 integrin subunit.

Gross anatomic analysis of the small intestine of the mi/mi animal did not show any difference in Peyer’s patch numbers or size compared with the wild type. The mi spleen, compared with the wild type, displays a lack of germinal center organization (data not shown), presumably due to depressed numbers of B cells. The Peyer’s patch sections show less differences between the two mouse strains.

The α4β7 integrin complex is critical for the migration of maturing T and B cells into the Peyer’s patches (36–38). Peyer’s patches from wild-type and mi mice were isolated, and single-cell lymphocyte suspensions were prepared and analyzed by FACS. Abs specific for α4 and CD19 were used (Fig. 8). Peyer’s patch lymphocytes obtained from the mi animals were about 50% CD19 positive compared with the wild-type sample, of which 65% of the cells were CD19 positive. Virtually all of the isolated lymphocytes expressed the α4 chain. These data indicate that the mi animal possesses fewer B cells than its wild-type counterpart, but that the relative distribution of such cells within the mutant is the same as wild type (spleen vs Peyer’s patch), which would not be expected if the B cell deficiency was due to a lack of α4 expression.
Discussion

This report has detailed a reanalysis of the nature of the mi mutation upon the differentiation of bone marrow-derived cells. As has been amply demonstrated, mice possessing this mutation show depressed numbers of all types of MC. This observation had been previously attributed to a lack of c-Kit expression on the surface of MC due to presumed Mi transcriptional control of the c-Kit gene. However, we could readily differentiate MC in SCF from bone marrow and spleen cultures obtained from mi mice, could demonstrate the cell-surface expression of the c-Kit tyrosine kinase receptor in mi-derived MC using a variety of different anti-c-Kit Abs, and could show that transcript levels for the c-Kit mRNA between the 1/1, 1/mi, and mi/mi MC were virtually the same. Our genotyping assay of such cells using the mi deletion as an internal marker confirmed the genetic background of such mice. It is our conclusion that c-Kit expression is not appreciably depressed in the mi mouse and that c-Kit is not regulated by Mi in MC. This conclusion is supported by the work of others who have been unable to demonstrate that the c-Kit gene promoter sequences are recognized by Mi (13). Other reports indicate that c-Kit and Mi are within a signal transduction pathway where the activation of c-Kit (via SCF) leads to phosphorylation of Mi and subsequent transcriptional up-regulation of M box-dependent genes (22). However, because we can readily develop mi/mi MC in SCF-only cultures, c-Kit functions to regulate more than just the Mi pathway. Because MC express Mi (39), there must be genes, such as MMCP-6, that are dependent upon Mi transcriptional control.

One recent hypothesis for the lack of MC in mi mice suggested that such animals lack a4 gene expression, which would limit the appropriate migration of MC into peripheral tissues. Transcript analysis of maturing mi/mi mast cells suggested that the a4 gene was under Mi gene control and that an atypical M box in the a4 gene promoter was responsible (32). Chimeric animals obtained from blastocysts derived from recombinase-activating gene-1 knockout mice and a4-deficient embryonic stem cells demonstrated a lack of mature B cells in the periphery and a lack of lymphoid morphology including the absence of definable Peyer’s patches (34). Thus, if the mi animal did lack expression of a4, it could help explain the lack of MC and B cells, as described in this report. We have previously described how integrin subunit expression can vary depending upon the age and cytokine environment of the MC, suggesting that tissue localization/homing signals are influenced by such differentiation triggers (24).

FIGURE 5. Absence of maturing B cells in mi bone marrow. Bone marrow cells were obtained from mi (mi/mi) and wild-type animals (+/+) stained with the denoted Abs (dark peaks) or the relevant isotype control (light peaks), and analyzed by FACS. The Abs used against the specific markers are noted on the horizontal axis.

![FIGURE 5](https://example.com/fig5)

FIGURE 6. Diminution of mature B cells in the mi spleen. Splenocytes were obtained from mi (mi/mi) and wild-type (+/+) animals and analyzed for the presence of the cell-surface markers via FACS. The dark peak indicates specific staining, the light curve is the nonspecific isotype control. Abs used against the surface receptors are denoted on the horizontal axis.

![FIGURE 6](https://example.com/fig6)

FIGURE 7. Integrin subunit a4 expression in mi tissues. The a4 integrin subunit transcripts were measured via RT-PCR (25 cycles) using transcripts derived from tissues samples from a mi animal (mi/mi) and a heterozygote animal (+/mi). Samples analyzed were spleen (S), thymus (T), intestine including Peyer’s patches (I), and bone marrow (B). b-actin controls (15 cycles) from each reaction are shown below.

![FIGURE 7](https://example.com/fig7)
We examined the expression of $\alpha_4$ in the $mi$ mouse. Our analyses of the $mi$ animals did not show any difference in $\alpha_4$ expression in the relevant tissues and by lymphocytes. In addition, the $mi$ mouse spleen and Peyer’s patches possessed mature B cells, albeit at lower levels than the wild type, which would not be expected for an $mi$-dependent $\alpha_4$ deficiency. One explanation to account for the previously published results (32) may be that the $\alpha_4$ gene is under Mi control in MC but not in B cells. Alternatively, the tissue culture-derived $mi$ MC may have turned off expression of the $\alpha_4$ gene because such cells are more lineage committed than their wild-type counterparts due to the decreased numbers of MC precursors in the $mi$ animals.

Because we could not readily account for the lack of MC in the $mi$ animal due to an intrinsic defect of that cell, we took the opposite approach. This alternative scenario would suggest that the paucity of MC number is due to a depressed number of MC precursors within the bone marrow, which in turn would give rise to fewer, yet functional mature, MC, analogous to those that we can derive in tissue culture. Such a precursor-deficiency model could also account for the depressed numbers of NK and B cells in the $mi$ animal. The osteopetrotic $mi$ femur bone has reduced marrow volume because of increased internal bone mass (40, 41). Such phenotypes can also be seen for other mutations including Pu.1 knockout animals that abrogate osteoclast and macrophage differentiation (42) and the rat microphthalmia blanc mutation in which the osteopetrosis is mild and transient (43, 44). Interestingly, only those mutations in the MITF gene product that are dominant negatives, such as $mi$, give rise to osteopetrosis. This observation was explained in part by a recent report in which Mi/TFE3 heterodimers (and by inference TFE3 homodimers) have been implicated as critical for osteoclast differentiation (45). Rats that do not express any Mi protein due to a large chromosomal deletion still possess functional osteoclasts, presumably via TFE3 function. Mice that lack a functional TFE3 product, but do express wild-type Mi, also possess normal osteoclasts, presumably through Mi homodimers.

The key question raised in the analysis of the $mi$ bone marrow was whether that site is competent to maintain the normal complement of bone marrow precursors. CFU analysis indicated that not only is such marrow deficient for MC precursors but is virtually devoid of maturing B lymphocytes as well. This lack of B cells does not appear to be due to an intrinsic defect within lymphoid progenitors (giving rise to T, B, and NK cells) because the $mi$ thymus possesses the correct morphology and usual complement of T cell types, and mature T cells are found within the $mi$ spleen. In addition, the $mi$ mouse possesses mature B cells in the periphery, albeit at much reduced levels. The percentage reduction of peripheral mature B cells in the $mi$ animal is virtually the same as the percentage reduction of MC in the same animal. These data point to the marrow environment as being responsible for the diminution of such cells in the animal. Although the physical space within the $mi$ bone marrow is reduced due to the abnormal bone mass, the marrow site does possess hemopoietic potential as evidenced by the numbers of Gr-1-positive cells and the capacity to develop a small number of IL-3-dependent CFU. Thus, there is the bone marrow cavity space to maintain B cell progenitors, but perhaps not the correct cytokine environment.

Recently, a knockout strain of mouse was developed that lacked functional osteoprotegerin ligand (OPGL) (46). OPGL, which is identical with TNF-related activation-induced cytokine (TRANCE) and receptor activator of NF-κB ligand (RANKL), is a member of the TNF family and is recognized by the TNF-receptor family member RANK, which is expressed on dendritic cells, T cells, and hemopoietic precursor cells (47–50). Mice lacking OPGL show severe osteopetrosis and completely lack osteoclasts. These mice also demonstrate impaired thymocyte and thymus development, a relative decrease in B220$^+$ B cells in the spleen, and no peripheral lymph nodes: Peyer’s patches were evident and the splenic architecture was normal. The physical analysis of the OPGL-deficient animal is similar, but clearly not identical, to that observed for the $mi$ animal. It is not known if OPGL is under Mi gene control; however, M box sequences are not evident within the gene’s promoter region (51). Interestingly, both the OPGL-deficient and $mi$ animal possess decreased numbers of peripheral B cells, which suggests severe osteopetrosis alters the bone marrow microenvironment in such a way as to compromise B cell development in that location.

A simple model to explain the data presented here for the $mi$ animal would be to suggest that a key precursor cell growth factor is absent in such animals. Such a factor would presumably be under Mi gene control and expressed by the marrow stromal cells; its absence could give rise to the lack of precursor cells for B and NK cells and MC at that site. Alternatively, as also seen for the OPGL animal, the lack of functional osteoclasts might cause an exclusion from the marrow space of the cell, which is the source of such a cytokine. We would not anticipate this factor to be critical in the development of most granulocyte lineages because the Gr-1-positive population in the marrow appears to be unaffected by the $mi$ mutation. At this time, we do not know the identity of such a growth factor. Current screens for the aberrant expression of known precursor growth factors/cytokines have not identified any candidate molecules. We are currently in the process of expanding this candidate factor screen to include a wider variety of known molecules and to also use gene subtraction/differential expression techniques to screen bone marrow libraries from $mi/mi$ and $+/+$ mice. Once such a candidate gene is found, its gene product could be used in trans to complement the $mi$ mutation and thus verify its mode of action.

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![FIGURE 8.](http://www.jimmunol.org/issue_html/2006/177/6677f8.jpg)
References


