B and T Cells Are Not Required for the viable motheaten Phenotype

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Summary

Hematopoietic cell phosphatase (HCP), encoded by the hph gene, (also called PTP1C, SHP, SH-PTP1, and PTPN6) is deficient in motheaten (me/me), and the allelic viable motheaten (me~/me) mice. Since HCP is expressed in many cell types and protein phosphorylation is a major mechanism of regulating protein function, it is not surprising that the motheaten phenotype is pleiotropic. It is commonly thought that immune system involvement causes this disease. If so, the motheaten disease ought to be alleviated when the recombination activation gene-1 (RAG-1) is disrupted because there will be no V(DJ) rearrangement and thus impaired development of B and T cells. We bred homozygous, double-mutant me~/me~/RAG-1-/- mice and found that, in fact, they developed full-blown motheaten inflammatory disease, including pneumonitis, alopecia, inflamed paws, and splenomegaly with elevated myelopoiesis. Thus, except for autoantibodies, the motheaten phenotype does not depend on the presence of B and T cells. This observation cautions the use of motheaten mice as a model of autoimmune disease.

Materials and Methods

Mice. The founding C57BL/6j +/+ me~ mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and the founding RAG-1-/- mice were kindly provided by S. Tonegawa (Massachusetts Institute of Technology, Cambridge, MA). Mice were maintained at the animal facility of the Ontario Cancer Institute (Toronto), and kept in microisolator housing units (Lab Products, Maywood, NJ) in rooms equipped with the Hepa-filtered air handling system. Cages, bedding, and water were sterilized by autoclaving, and food was sterilized by γ-irradiation. Mice were handled by aseptic forceps or aseptic gloves.
Figure 1. Generation of mice with various genotypes at the me and RAG-1 loci. Since me/me mice are sterile, mice heterozygous at the me locus were used for breeding. The founding+/me and RAG-1-/- parents were from the C57BL/6J and 129/Sv × CD1 backgrounds, respectively. The F1+/me/RAG-1-/- mice were back-crossed to the RAG-1-/- parent to generate the F2 generation. The F2 mice were then intercrossed to generate the F3 generation, which were obtained by the following crosses: (1) cross among the +/me/RAG-1-/- mice, (2) cross among the +/me/RAG-1-/- mice, and (3) cross between the +/me/RAG-1-/- mice and +/me/RAG-1-/- mice. The F3 generation consisted of mice of all possible genotypes, i.e., the normal control mice (i.e., mice that have at least one wild-type allele at each of the me and RAG-1 loci), the RAG-1-/- control mice (i.e., mice that are homozygous for the RAG-1 null mutation and have at least one wild-type allele at the me locus), the me/me control mice (i.e., mice that are homozygous for the me mutation and have at least one wild-type allele at the RAG-1 locus), and the double-mutant mice (i.e., mice that are homozygous for both the me mutation and RAG-1 null mutation).

Genetic Testing. The strategy used for typing the me locus was to amplify the region of the genome containing the me mutation by PCR and then to examine the amplified products by single-strand conformation polymorphism (SSCP) analysis. The primers used for amplifying a 167-bp fragment in which the me mutation is located were 5'-TGTCATCGTCATGACTACC-3' and 5'-TCAGGCTTGGCAGCAAACTCG-3'. The latter primer was kinased with [33p]ATP. PCR conditions used were 15 s at 94°C, 15 s at 54°C, and 15 s at 72°C for 30 cycles. The amplified products were then subject to SSCP analysis: a portion of the PCR product was boiled for 3 min, quickly chilled on ice, loaded on an MDE (J.T. Baker Chemical Co., Phillipsburg, NJ) gel, and run at 3 W for 19 h. The gel was dried, and the DNA was visualized by autoradiography.

The RAG-1 locus was typed by PCR of the region of the genome containing the disruption in RAG-1. The primers used for amplifying an 809-bp RAG-1 fragment present only in the wild-type allele were 5'-ATCTCTGCTGCAATATGCGA-3' and 5'-AACCTCTTCTTTCATGGATCTT-3'. The primers used for amplifying a 477-bp neo fragment present only in the RAG-1 disrupted allele were 5'-ATGATTAAACAGTGGATTCGC-3' and 5'-TCCAGATCATCCTGATGC-3'. The PCR conditions used were 15 s at 94°C, 15 s at 54°C, and 30 s at 72°C. The extension time was increased by 3 s per cycle for a total of 30 cycles. The amplified DNA was subjected to agarose gel electrophoresis and then visualized by fluorescein of ethidium bromide under ultraviolet light.

Flow Cytometry. Cells taken from 5-wk-old mice were stained with FITC-conjugated anti-IgM heavy chain (33.60) (16), FITC-conjugated anti-CD4 (RM4-5; PharMingen, San Diego, CA), PE-conjugated anti-CD8oL (53-6.7; PharMingen), and PE-conjugated anti-Mac-1 (MI/70; Biosource International, Camarillo, CA) antibodies separately. Briefly, 2 X 10^6 cells were incubated with the appropriate antibodies (1 μg/10^6 cells) in 100 μl PBS plus 5% FCS (GIBCO BRL, Gaithersburg, MD) for 20 min on ice. The

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stained cells were then washed with PBS plus 5% FCS, and were analyzed by flow cytometry.

*Histological Analysis.* Tissues were taken from mice of various ages (from 1 to 15 wk). Cross-sections of 4–8-μm thick were obtained from wax-embedded tissue samples and stained with hematoxylin and eosin. Representative sections of the different tissues are shown in Figs. 5 and 6.

*Statistical Analysis.* The statistical significance of differences
between sample means was based on Cox regression analysis (for survival analysis), chi square \(X^2\) test (for genotype analysis), and Fisher's exact test (for all other analyses). In all tests, differences are treated as significant when \(P < 0.05\).

### Results

**Generation and Analysis of Mutant Mice.** The breeding scheme and designation of the various control mice are described in Fig. 1. We analyzed 30 informative litters. The genotype of each mouse was determined by DNA PCR and SSCP analysis for the \(me^v\) locus and by PCR for the \(RAG-1\) locus (Fig. 2 a). When mice were killed, the genotypes were verified by flow cytometry, analyzing spleen cells with antibodies recognizing the IgM heavy chain (\(\mu\)), CD4, CD8, and Mac-1 (Figs. 2 b and 6 e). Litter sizes ranged from 1 to 12, and the frequencies of the various genotypes were not significantly different from the expected Mendelian frequencies, i.e., \(0.150\) of the mice were \(me^v/me^v\) control (expected frequency = \(0.149\), \(n = 107\), \(\chi^2 P > 0.900\)), and \(0.194\) of the mice were double mutant (expected frequency = \(0.155\), \(n = 160\), \(\chi^2 P > 0.250\)). The expected frequencies are calculated by taking into account of the various types of crossing that were carried out to generate a given genotype. For example, double-mutant mice were obtained from three types of crossing (see Fig. 1), i.e., (1) \(+/me^v. RAG-1^{+/+} \times +/me^v. RAG-1^{+/+}\) with a \(P = 0.0625\) for generating the double mutant; (2) \(+/me^v. RAG-1^{+/+} \times +/me^v. RAG-1^{+/+}\), with a \(P = 0.25\) for generating the double mutant; and (3) \(+/me^v. RAG-1^{+/+} \times +/me^v. RAG-1^{+/+}\), with \(P = 0.125\) for generating the double mutant. A total of 35 mice were generated from the type 1 crossing, 56 mice from the type 2 crossing, and 69 mice from the type 3 crossing. The expected frequency of generating the double mutant is therefore \([35](0.0625) + (56)(0.25) + (69)(0.125)] / 160\) = 0.155. There was no significant difference between this expected frequency and the observed frequency of \(0.149\) of the mice were \(me^v/me^v\) control (ex-pected frequency of \(me^v/me^v\) control do not differ significantly in the probability of survival at any given time (see Fig. 3).

### Table 1. Characteristics of the motheaten and RAG-1 Mutants*

| Characteristic | Motheaten \(+/^v\) RAG-1 \(^{+/+}\) | Motheaten \(+/^v\) RAG-1 \(^{+/+}\) | Motheaten \(+/^v\) RAG-1 \(^{+/+}\) | Motheaten \(+/^v\) RAG-1 \(^{+/+}\) |
|---------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Median life span \(\text{d}\) | >275 \(n = 49\) | 190 \(n = 80\) | 72 \(n = 16\) | 97 \(n = 31\) |
| Skin: focal alopecia | \(0/48\) | \(9/48\) | \(7/13\) | \(9/23\) |
| Extremities: swelling and lesions | \(0/48\) | \(0/79\) | \(11/13\) | \(19/29\) |
| Lung abnormalities | \(4/9\) | \(8/12\) | \(5/7\) | \(12/12\) |
| Inflammation | \(4/9\) | \(8/12\) | \(3/7\) | \(11/12\) |
| Intraalveolar erythrocytes | \(1/9\) | \(2/12\) | \(3/7\) | \(5/12\) |
| Mφ with crystalline material | \(0/9\) | \(0/12\) | \(0/7\) | \(8/12\) |
| Spleen: splenomegaly | \(1/8\) | \(1/10\) | \(7/7\) | \(7/10\) |

* \(+/-\) denotes at least one wild-type allele; \(v\) denotes the allele carrying the viable motheaten mutation.

†Survival analyses indicate that the double-mutant and the \(me^v/me^v\) control do not differ significantly in the probability of survival at any given time (see Fig. 3).

\(\Delta\) Difference between the \(me^v/me^v\) control and double-mutant is not significant \((P = 0.299\) in Fisher's exact test). 

\(\Delta\) Difference between the \(me^v/me^v\) control and double-mutant is significant \((P = 0.047\) in Fisher's exact test).

\(\Delta\) Lungs scored abnormally if they contained one or more of the three pathological characteristics, i.e., inflammation, intraalveolar erythrocytes, and Mφ with crystalline material.

** Difference between the \(me^v/me^v\) control and double-mutant is significant \((P = 0.038\) in Fisher's exact test).

\(\Delta\) Difference between the \(me^v/me^v\) control and double-mutant is significant \((P = 0.006\) in Fisher's exact test).

\(\Delta\) Spleenomegaly was scored by visual inspection.

Between sample means was based on Cox regression analysis (for survival analysis), chi square \(X^2\) test (for genotype analysis), and Fisher's exact test (for all other analyses). In all tests, differences are treated as significant when \(P < 0.05\).
Inflammatory Lesions in the Extremities. Both the me\textsuperscript{e}/me\textsuperscript{e} control and double-mutant mice developed the characteristic inflammation in the paws: extensive swelling and open pustular lesions. The paw lesions typically appeared between 5 and 10 d after birth, and the severity of these swellings and lesions was of the same magnitude (Fig. 4, e–g). The frequencies of paw deformities in the me\textsuperscript{e}/me\textsuperscript{e} control (11 of 13) and double-mutant mice (19 of 29) were similar (Table 1). The reason for incomplete penetrance of these symptoms is unknown, but may be caused by a variation in the time of onset of the disease.

Lung Abnormalities. Lung abnormalities characteristic of pneumonitis were present in \(~70\)% of the me\textsuperscript{e}/me\textsuperscript{e} control and in all double-mutant mice examined (Table 1). These pneumonitis-like characteristics were the likely cause of death of these mice during the first few months of life (Table 1 and Fig. 3). Histological analysis revealed severe inflammation in the lungs of both me\textsuperscript{e}/me\textsuperscript{e} control and double-mutant mice (Table 1 and Fig. 5 c–e). A proportion of the normal control and RAG-1\textsuperscript{-/-} control mice had some inflammation (Table 1), but it was a much milder form than that in the lungs of the me\textsuperscript{e}/me\textsuperscript{e} control and double-mutant mice (Fig. 5, a and b). A large amount of crystalline material, which appears to be associated with degradation of erythrocytes (10), is present inside the alveolar macrophages of the double-mutant mice. This abnormality has also been observed by Shultz (10), who reported crystalline material in macrophages in the C57BL/6J me\textsuperscript{e}/me\textsuperscript{e} mice. The reason for their absence in the me\textsuperscript{e}/me\textsuperscript{e} control in the present study is unknown. Since the me\textsuperscript{e}/me\textsuperscript{e} control mice in our study are of a mixed genetic background (see Fig. 1), however, strain-specific factors may modulate the severity of the lung abnormalities. No crystalline material was detected in the lung sections of the RAG-1\textsuperscript{-/-} control or normal control (Table 1). These results lead to the conclusion that in the absence of B and T cells, me\textsuperscript{e}/me\textsuperscript{e} mice develop severe hemorrhagic pneumonitis.

Extramedullary Myelopoiesis. Elevated myelopoiesis in the spleen of the C57BL/6 me\textsuperscript{e}/me\textsuperscript{e} mice results in splenomegaly (10). In the present study, the incidence of splenomegaly was similar in the me\textsuperscript{e}/me\textsuperscript{e} control and double-mutant mice (Table 1). The spleens of these mice generally had no organized white pulp (Fig. 6 a–d) and contained more than fivefold higher numbers of myeloid cells, as detected by anti-Mac-1 antibody, than the normal control and RAG-1\textsuperscript{-/-} control mice (Fig. 6 e).
Discussion

Since the RAG-1 mutation used in the present study completely and specifically blocks the development of B and T cells, our results indicate that except for the production of autoantibodies, the manifestations of the motheaten inflammatory disease occur in the absence of the antigen-specific arm of the immune system. Contrary to our findings, Shultz and Sidman (18) reported that the me/me scid/scid mice had a threefold increase in life span as compared to the me/me' mice. This difference in life span might be because the effects of the scid mutation are not limited to B and T cells (19, 20). Moreover, earlier studies using mouse mutations that partially block either B or T lymphopoiesis are consistent with our analysis of the completely B- and T-deficient me/me'RAG-1−/− mutant mice. Scribner et al. (21) and Shultz (14) observed that neither the presence of the xid mutation, which impairs the production of...
Figure 5. Histological analysis of the lungs of the double-mutant mice reveals severe inflammation. The lungs of (a) normal control and (b) RAG-1−/− control mice are relatively normal. (c) The lungs of me+/me control mice typically have inflammation characterized by interstitial fibrosis, resulting in greatly reduced air space. (d) The lungs of double-mutant mice typically have inflammation characterized by a large number of alveolar macrophages containing crystalline material and infiltration of granulocytes. (e) The presence of intraalveolar erythrocytes are also common in the double-mutant mice. Histological sections were obtained from 3–6-wk-old mice. X40.

CD5+ B cells, nor the presence of the nude mutation, which blocks T cell development, prevented the manifestations of the motheaten phenotype, respectively. These studies, although informative, did not generate a definitive conclusion about the role of B and T cells in the motheaten disease, since neither the xid nor the nude mutation ablates both functional B and T cells (22–25). However, our analysis taken together with these previous studies argues that the motheaten phenotype does not require B and T cells. Altogether, these observations call into question the use of me+/me and me+/me mice as a paradigm of autoimmune disease.
Figure 6. Extramedullary myelopoiesis of the me/ me control and the double-mutant mice. (a) The white pulps in the spleen of the normal control are well structured. No organized white pulp is observed in the (b) RAG-1−/− control, (c) me/ me control, or (d) double-mutant mice. (e) Large absolute numbers of cells stained positive for Mac-1 are present in the me/ me control and double-mutant mice. In comparison, the spleens of the normal control and RAG-1−/− control have low absolute numbers of the Mac-1+ cells, although the frequency of these cells is high in the RAG-1−/− control because of the absence of B and T cells. Analysis was carried out on live cells (see Fig. 2 b). The error bars of the histograms are SE values from two experiments. Spleens were obtained from 3–6-wk-old mice for analysis.

ease are not known. Infiltration of the affected mouse organs by monocytes, macrophages, and granulocytes suggest that these cell types are the primary effectors of inflammation in the me/ me mice. Consistent with this notion is the observation that treatment with an antibody against CD11b, a subunit of Mac-1 whose expression is restricted to mononuclear phagocytes, granulocytes, and NK cells (26, 27) inhibits inflammation in me+ bone marrow chimeric mice (11). Since the NK cells of me+ mice have virtually no activity and are present at only 30–50% of the normal levels (28), the CD11b study suggests that myeloid cells are responsible for the inflammatory disease of the me/ me mice (11).

Cells of nonhematopoietic origin may also contribute to the motheaten diseases. Although HCP is expressed predominantly in bone marrow, thymus, and hematopoietic cell lines (3, 4, 29), it is detected in low abundance in a few epithelial cell lines and nonhematopoietic cancer cell lines (4, 29). Mutating HCP in a cell-specific manner should delineate the cell types that are important in the manifestation of the motheaten disease.
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