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Genetics

HLA- and H-2-associated variations of intra- and extracellular magnesium content

(major histocompatibility complex/genetics/human/mouse)

JEAN G. HENROTTE*†, MARIKA PLA‡, AND JEAN DAUSSET§

*Centre National de la Recherche Scientifique, Faculté de Pharmacie, Paris, France; ‡Institut National de la Santé et de la Recherche Médicale U 93, Hôpital Saint-Louis, Paris, France; and §Centre d'Etude du Polymorphisme Humain, Hôpital Saint-Louis, Paris, France

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ABSTRACT **Erythrocyte and plasma magnesium (EMg, PMg) levels have been shown to be genetically controlled in human and mouse. The possible association of these genetic factors with the major histocompatibility complex (MHC) (HLA and H-2) was investigated. Among unrelated adult male blood donors, HLA-B35 carriers have PMg ($P < 0.01$) and EMg ($P < 0.0005$) levels lower than those of noncarriers, while HLA-B38 carriers exhibit a significant ($P < 0.05$) increase of both PMg and EMg levels when compared to the other individuals. Furthermore, HLA identical sibs have EMg values more similar than those of HLA different sibs. In the mouse, erythrocyte ($P < 0.001$), plasma ($P < 0.001$), liver ($P < 0.03$), and spleen ($P < 0.04$) Mg contents vary significantly according to H-2, with higher values being found in H-2^k than in H-2^q or H-2^b congenic strains. However, non-MHC genes also have an influence on erythrocyte ($P < 10^{-10}$), plasma ($P < 10^{-10}$), spleen ($P < 10^{-5}$), and kidney ($P < 10^{-6}$) Mg contents as shown by differences between H-2 identical strains, which differ by the C3H and B10 genetic backgrounds. In conclusion, genetic factors controlling intra- and extracellular Mg levels are composed of at least three components: MHC (HLA and H-2)-associated genes, non-MHC genes, and tissue factors modulating the respective importance of the first two sets of factors. The mechanisms underlying this genetic system are discussed.**

The comparative study of various ethnic groups revealed the occurence of significant ($P < 0.001$) variations of their mean erythrocyte magnesium (EMg) content (1, 2). The magnitude of these differences and their relative stability withstanding variations in the environment led to formulate the hypothesis (1) of a genetic control of Mg level in human erythrocytes. This hypothesis was confirmed by twin and family studies (3–5), which also revealed a similar control for plasma Mg (PMg) concentrations. In mice, interstrain comparisons led to the same conclusions (6).

Some of the involved genes are associated with the major histocompatibility complex (MHC) as shown by variations of EMg and PMg levels according to HLA phenotypes in humans (7–9) and H-2 haplotypes in mice (6). Among 351 unrelated male blood donors, 57 subjects carrying the HLA-B35 antigen ($B35^+$) exhibited lower EMg values ($P < 0.001$) than the remaining 294 noncarriers ($B35^-$) (7). In mice, 372 adult males belonging to 13 H-2 congenic strains with either a C3H or a B10 genetic background revealed large and significant differences in EMg and PMg levels. More specifically, on both backgrounds, the strains carrying H-2^q had lower plasma and erythrocyte Mg levels than the strains carrying H-2^k ($P < 10^{-4}$), although in general the C3H background strains exhibited lower values than the B10 background strains ($P < 10^{-7}$) (6).

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These results prompted us to further study relationships between intra- and extracellular Mg contents and MHC. In humans, additional data on blood Mg levels were obtained from unrelated blood donors of both sexes and new data from families of known HLA groups. In mice, Mg contents were determined in soft tissues (liver, spleen, thymus, kidney, and skeletal muscle) of six H-2 congenic strains carrying H-2^b, H-2^k, and H-2^q haplotypes on both C3H and B10 genetic backgrounds. These results are presented and compared with those previously obtained.

MATERIALS AND METHODS

Blood Donors. Blood was sampled in the morning before breakfast from 96 healthy male volunteers (25–69 years old) and from 112 females at a fertile age (21–51 years old), all unrelated French Caucasians living in the Paris area. Blood was also sampled from 147 male and 133 female siblings belonging to 68 families, with an average of 4 children per family. Their age distribution, similar in both sexes, was the following: <20 years, 32%; ≥20 years and <30 years, 56%; ≥30 years, 12%. The blood collected on heparin was immediately centrifuged at 1000 × *g* for 10 min to separate plasma and erythrocytes. The latter were recentrifuged and the top part of the erythrocyte column (buffy coat and younger erythrocytes) was discarded.

Animals. Organs were dissected from 107 male mice of six H-2 congenic strains carrying H-2^b, H-2^k, and H-2^q haplotypes with both C3H (C3H.B10, C3H, C3H.Q) and B10 (B10, B10.BR, B10.G) genetic backgrounds. The animals originated from two different generations of the same colony (INSERM U 93); the first one (experiment 1) included 45 mice (80 ± 12 days) and the second (experiment 2) had 62 mice (118 ± 17 days), sacrificed about 1 year apart. Details of the composition of the subgroups are given in Fig. 3. The animals of the six tested strains were randomized; the liver, spleen, thymus, kidneys, and skeletal muscle (gastrocnemius) were sampled and blotted on ash-free filter paper to remove excess blood. The PMg and EMg data of the same strains, obtained on different series of mice, have been published (6).

Organ Weight and Water Percentage. The organ fresh weight was determined as well as the dry weight after they were dried for 8 days at 80°C and maintained under vacuum (<15 mmHg) until constant values were reached. The weight of water was expressed as percent total weight. The dry material was converted to ash by nitric acid and hydrogen peroxide digestion at 80°C–120°C until complete mineraliza-

Abbreviations: MHC, major histocompatibility complex; EMg, erythrocyte magnesium; PMg, plasma magnesium.

†To whom reprint requests should be addressed at: Centre National de la Recherche Scientifique, Faculté de Pharmacie, 4, Avenue de l'Observatoire, 75006 Paris, France.

tion was achieved. The residues dissolved in distilled water were used for Mg determinations.

Mg Determinations. Mg determinations were done by flame absorption spectrophotometry following the recommendations of Stendig-Linberg *et al.* (10). The detailed procedure has been described (7, 11). The coefficient of variation was equal to 1% (7, 11), emphasizing the high reproducibility of Mg determinations. Values are expressed in mM for blood Mg concentrations (which means mmol per liter of erythrocytes or plasma) and in mmol per kg fresh or dry weight for soft tissues.

HLA Determinations. HLA-A, -B, and -C antigens were determined in all subjects, and HLA-DR was determined in most of them. The following HLA-B specificities were determined: B5, B7, B8, B13, B14, B17, B18, B21, B22, B27, B35, B37, B38, B39, B40, B41, B44, B45, B62, B63. HLA-Bw4 and -Bw6 specificities were also determined in the majority of cases.

Statistical Analysis. The significance of differences between means compared two by two was determined by Student's t test. When the F ratio of variances was significantly different from 1, at the level of $P < 0.05$, Cochran's t test was used instead of Student's t test (12). The mouse data were submitted first to one-way variance analysis to evaluate the H-2 effects on each genetic background (C3H or B10) separately; they were then subjected to two-way analysis of variance to test the overall effects of H-2 and non-H-2 genes and the interaction between these two genetic factors. Analyses were done separately on the data of experiments 1 and 2. The combined probabilities for the two experiments were then computed according to Snedecor (12). Unbiased estimates of strain mean differences (taking into account unequal subclass numbers in experiments 1 and 2) were calculated by the method of fitting constants (12). χ^2 values were computed for comparing percentages. The intraclass correlation coefficients (r_I) were calculated for heritability estimation (13).

RESULTS

Blood Mg of Unrelated Subjects. The 96 male blood donors tested in the present series yield results similar to those of the 351 subjects examined previously (7). The two sets of data have therefore been pooled for the following analysis. $B35^+$ subjects have a mean EMg significantly lower ($P < 0.01$) than $B35^-$ individuals (Table 1). However, it has been suggested (14) that the HLA-B35 specificity can be subdivided in two groups: the most frequent one (≅90%) is associated with the specificity Bw6; the other is associated with the specificity Bw4. The $B35^+$ blood donors were therefore subdivided into $B35^+Bw6^+$ and $B35^+Bw4^+$. $B35^+Bw4^+$ subjects have slightly higher PMg and EMg mean values than $B35^+Bw6^+$ individuals (Table 1). The distributions of EMg values show significant differences ($P < 0.04$): 69% of $B35^+Bw4^+$ and only 38% of $B35^+Bw6^+$ individuals exhibit values higher than the overall mean (2.1 mM). The $B35^+Bw6^+$ subjects have PMg and EMg mean values significantly lower than those of $B35^-$ subjects ($P < 0.01$ and $P < 0.005$, respectively; Table 1). The PMg and EMg variances are also smaller in the $B35^+Bw6^+$ group than in the $B35^-$ population ($P < 0.05$), indicating a greater homogeneity in the former group than in the latter. The distribution of EMg values above and below 2.1 mM is also significantly different in these two groups ($P < 0.0005$; Fig. 1 *A* and *C*). The distribution of PMg (data not shown) yields similar results with a lower level of significance ($P < 0.01$). In contrast, B38 carriers ($B38^+$) have higher PMg and EMg values than noncarriers ($B38^-$) and than $B35^+Bw6^+$ individuals (Table 1 and Fig. 1*B*). Blood donors with other HLA-B antigens exhibit PMg and EMg contents that differ little from the general population mean in agreement with our previous findings (7). Likewise, no significant variations of PMg and EMg were found related to HLA-A, -C, or -DR polymorphism.

Among female blood donors, no significant differences were found for PMg and EMg (mean ± SEM) between $B35^+$ (0.76 ± 0.009 and 2.08 ± 0.037 mM) and $B35^-$ individuals (0.77 ± 0.005 and 2.09 ± 0.022 mM).

Blood Mg in Sibships. In each family, the child with the lowest EMg value was arbitrarily considered as propositus, whatever the sex (15). Children were classified into three groups according to their HLA antigens: identical, semi-identical, or different from those of the propositus. The distribution of values was studied separately in each sex. The

Table 1. Means (±SEM) of PMg and EMg levels (mM) in various groups of male blood donors classified according to their HLA-B specificities

Compared groups (n)	PMg		EMg	
$B35^+Bw6^+$ (55)	0.76 ± 0.005	NS	2.08 ± 0.025	NS
$B35^+Bw4^+$ (13)	0.78 ± 0.017	**	2.18 ± 0.052	**
$B35^+$ total (82)	0.77 ± 0.005	NS	2.11 ± 0.020	**
$B35^-$ total (365)	0.78 ± 0.003		2.17 ± 0.012	
$B38^+$ (19)	0.80 ± 0.009	*	2.26 ± 0.044	*
$B38^-$ (428)	0.78 ± 0.002		2.15 ± 0.011	
$B35^+Bw6^+/B38^-$ (54)	0.76 ± 0.005	***	2.08 ± 0.025	***
$B35^-/B38^+$ (16)	0.81 ± 0.009	**	2.29 ± 0.047	**
$B35^-/B38^-$ (349)	0.78 ± 0.003		2.16 ± 0.012	

The $B35^+$ total group includes 55 $B35^+Bw6^+$, 13 $B35^+Bw4^+$, and 14 $B35^+$ individuals with undetermined Bw4 or Bw6 specificities. NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

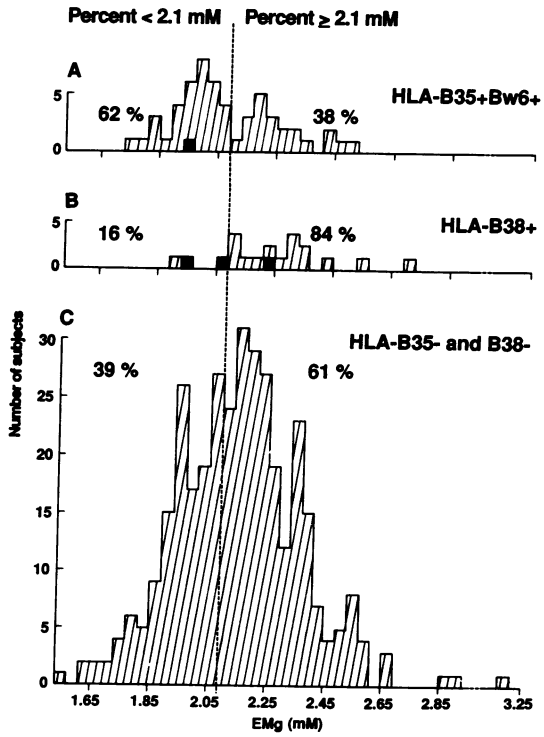

FIG. 1. Distribution of EMg levels in adult male blood donors classified according to their HLA specificities. (*A*) Fifty-five HLA-$B35^+Bw6^+$ individuals. (*B*) Nineteen HLA-$B38^+$ individuals. (*C*) Three hundred forty-nine individuals carrying HLA-B specificities other than B35 and B38. The percentages of individuals above and below the general population mean (2.1 mM) are indicated. Significance of differences in percentage distribution: *A* versus *B* (P = 0.0003), *B* versus *C* (P = 0.05), *A* versus *C* (P = 0.0005) when three individuals with both HLA-B35 and -B38 specificities (solid area) are excluded; among them, only one is $B35^+Bw6^+$.

Table 2. Distribution of EMg levels in 68 sibships

	n	% individuals with EMg		
		Low	Medium	High
	Male sibs			
Propositi	34	68	23	9
HLA identical*	33	27	52	21
HLA semi-identical	54	22	50	28
HLA different*	26	38	15	46
	Female sibs			
Propositi	34	82	15	3
HLA identical	22	46	36	18
HLA semi-identical	48	39	44	17
HLA different	29	31	38	31

In each sibship, the subject with the lowest value is considered as propositus independent of the sex. The other sibs are classified into three groups according to their HLA antigens: identical, semi-identical, and different from those of the propositus. EMg levels: low, <2.05 mM; medium, ≥2.05 and <2.25 mM; high, ≥2.25 mM. *Distributions significantly different ($P = 0.01$).

HLA identical sibs have values much closer to those of the propositi than the HLA different sibs (Table 2). The distribution of values between HLA identical and HLA different sibs is significantly different ($P = 0.01$) among male children; the same tendency is observed among females but is not significant. No significant differences were observed for PMg.

Tissue Mg in Mice. Mg levels (mmol per kg fresh weight) and water content (% total fresh weight) were determined from five organs of mice in six H-2 congenic strains. Two independent experiments, although yielding somewhat different mean values, showed a striking parallelism. Therefore, the values of experiments 1 and 2 were pooled. Unbiased estimates of strain mean differences are given in Fig. 2 and reveal the existence of a general trend toward higher values

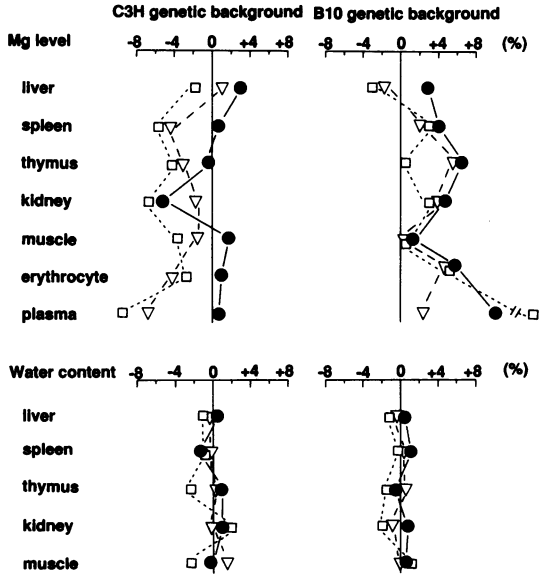

FIG. 2. Compared variations of blood and tissue Mg levels (*Upper*) and tissue water content (*Lower*) of male mice carrying H-2^b (□), H-2^k (●), and H-2^q (▽) haplotypes with either a C3H genetic background [13 C3H.B10 (H-2^b), 17 C3H (H-2^k), 22 C3H.Q (H-2^q)] or a B10 genetic background [16 B10 (H-2^b), 21 B10.BR (H-2^k), 18 B10.G (H-2^q)]. Differences between mean values of each strain and the general mean are expressed as percent of the general mean. The data used are the unbiased estimates of strain means computed from values of experiments 1 and 2 (in mmol of Mg per kg fresh weight and in water percentage), except blood data, which have been published (6). The PMg value of H-2^b mice on the B10 background (+28%) falls out of scale.

Table 3. Effect of H-2 and non-H-2 genes on blood and tissue Mg level and tissue water content

	Factors of variation				
	H-2				
Tissue	C3H	B10	Total	Non-H-2	Interaction
	Mg level				
Liver	NS	10^{-5}	0.03	NS	NS
Spleen	3×10^{-5}	NS	0.04	10^{-5}	NS
Thymus	NS	0.04	NS	0.02	NS
Kidney	NS	NS	NS	10^{-6}	NS
Muscle	NS	NS	NS	NS	NS
Erythrocyte	2×10^{-10}	NS	3×10^{-4}	10^{-11}	0.02
Plasma	3×10^{-5}	10^{-10}	7×10^{-4}	10^{-10}	2×10^{-11}
	Water content				
Liver	0.01	NS	0.05	NS	NS
Spleen	0.04	0.01	NS	5×10^{-5}	0.01
Thymus	0.01	0.01	0.004	NS	NS
Kidney	NS	2×10^{-6}	0.02	4×10^{-5}	6×10^{-4}
Muscle	NS	NS	NS	NS	NS

The combined probabilities given in this table were computed from the P values of experiments 1 and 2 calculated by two-way analysis of variance (H-2 and non-H-2 genes). The probabilities were also calculated by one-way analysis of variance to test separately the H-2 effect on the C3H and the B10 genetic backgrounds (columns 1 and 2). The calculations are based on the data presented in Fig. 2. NS, not significant.

in H-2^k than in H-2^q and H-2^b mice and higher in animals with a B10 than in those with a C3H genetic background. The statistical analysis of these data is indicated in Table 3. When Mg levels are expressed in mmol per kg dry weight instead of fresh weight, similar results are obtained; for example, these values are given for two organs (liver and spleen) in Fig. 3. For the sake of comparison, PMg and EMg values of the same strains, previously published (6), are also indicated in Fig. 2 and Table 3.

H-2-associated Mg variations are significant for liver, spleen, erythrocytes, and plasma; those appertaining to the genetic background (C3H or B10) are significant for spleen, thymus, kidney, erythrocyte, and plasma (Table 3). In most cases, variations of tissue water content follow a different pattern: H-2-associated variations are significant for thymus and kidney, and non-H-2 variations are significant for spleen and kidney (Table 3). Significant interactions (Table 3) result from the fact that H-2-associated variations are significantly different in the C3H and in the B10 genetic backgrounds, suggesting that the penetrances of the H-2 and the non-H-2 effects are interdependent.

Intraclass correlation coefficients (r_I) were calculated from the values of the six strains tested in experiment 2. These coefficients, which give a good estimate of heritability (12, 13), are high and significant for spleen and kidney Mg ($r_I \geq 0.4$; $P \leq 10^{-5}$); r_I cannot be safely estimated from the liver values since interstrain differences depend on the genetic background (Fig. 3 and Table 3).

DISCUSSION

The genetic control of erythrocyte and plasma Mg levels was previously shown to be a quantitatively important phenomenon: the heritability coefficients were high, equal to 0.92 for EMg and 0.72 for PMg in human (5) and to ≈0.60 for both EMg and PMg in mouse (6). The present data show that the heritability of mouse tissue Mg contents is also high. The genetic system involved is obviously polygenic and our results demonstrate clearly that at least one of the genes is directly or indirectly associated with the MHC. However, the

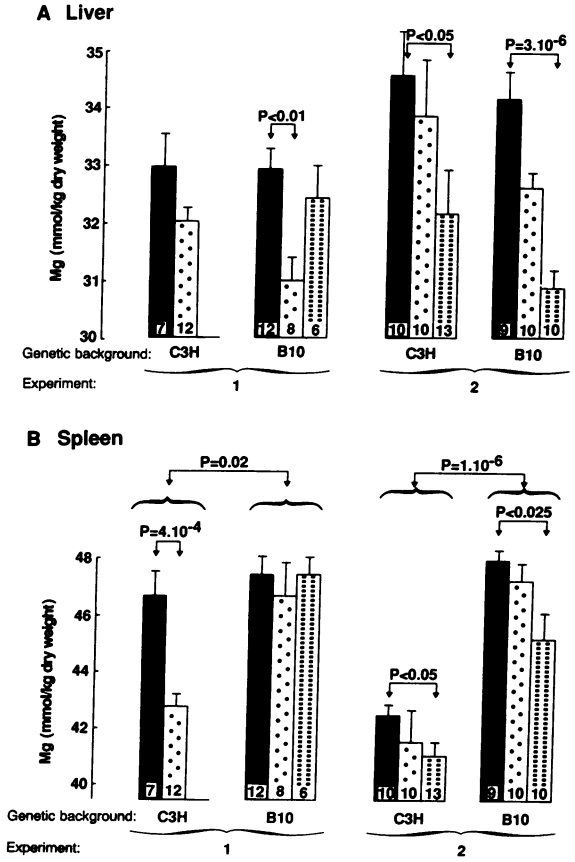

FIG. 3. Means and standard errors of Mg content (mmol per kg dry organ weight) determined in the liver (*A*) and in the spleen (*B*) from male mice carrying H-2^k (solid), H-2^q (dotted), and H-2^b (dashed) haplotypes with the C3H and B10 genetic backgrounds. Data are given separately for experiments 1 and 2. Numbers of animals used are given at the column base. *P*, significance of difference between means.

part of genetic variance associated with the MHC appears smaller than that of non-H-2 genes.

In humans, the precise contribution of HLA-associated factors to the genetic variability of blood Mg levels is difficult to assess, since environmental factors such as age (11, 16, 17), season (11), and dietary habits (4) also play a role in the overall variability of blood Mg. In women, there are additional causes of variability linked to ovarian activity: both PMg and EMg are lower in women at a fertile age than after menopause (11, 16, 17). Their blood Mg levels also fluctuate with menstrual cycle and oral contraceptive intake (18). This may explain that no significant variations of blood Mg appear to be associated with HLA, either in the group of unrelated female blood donors or in that of female sibs (Table 2). However, even if an HLA-associated variability is overcast by other factors in women, it nevertheless exists as in men. As a matter of fact, in the familial study, the difference observed among male sibs between HLA identical and HLA different sibs is the same whatever the sex of the propositus (data not shown). This means that the HLA-associated EMg variability is phenotypically apparent only in males but is present in both sexes. The data of Table 2 reveal another interesting feature: male sibs exhibit a similar proportion of low EMg values (<2.05 mM) whatever the HLA, identical or different from the propositus, suggesting that the low EMg level of these subjects is controlled by non-HLA genetic factors; in contrast, in the medium class interval (≥2.05 and <2.25 mM), the proportion of subjects drops from 52% in the HLA identical group to 15% in the HLA different one. This latter group shows, therefore, a somewhat bimodal distribution of EMg values, indicating the existence of two sets of genetic factors, one HLA and one non-HLA-associated.

In the mouse, the occurrence of MHC- and non-MHC-associated genes in the regulation of intra- and extracellular Mg contents is further demonstrated by the data of Table 3 and Figs. 2 and 3. In liver H-2, and in kidney non-H-2, factors are principally involved, while Mg values vary significantly according to both H-2 and non-H-2 factors in spleen, erythrocytes, and plasma. These findings might imply that the expression of non-MHC genes is tissue specific.

Genetic factors regulating PMg levels may exert their action via renal functions since kidneys are known to be the main organs involved in PMg homeostasis. High or low PMg levels might entail a general trend toward high or low intracellular Mg: higher values in mice with B10 than C3H background and higher values in H-2^k than in H-2^q and H-2^b mice. In humans, $B35^+$ subjects have both PMg and EMg values lower than $B35^-$ blood donors. However, the genetic correlation between PMg and EMg levels is not a tight one and has been estimated to be 0.27 (5). There is also little correlation between plasma and tissue Mg contents in mice (Table 3): in liver, Mg variations are almost exclusively H-2 dependent, while in plasma they are associated with both H-2 and non-H-2 factors. Genetic variations of PMg levels therefore cannot account for the whole variability of Mg levels in soft tissues and erythrocytes.

The strain differences in tissue Mg levels cannot be attributed to differences in water content since no correlations were found between these two variables (Fig. 2 and Table 3). Furthermore, when Mg content is related to dry weight (Fig. 3) instead of fresh weight, the differences between strains remain nearly the same. Thus, strain differences observed in tissue Mg levels most probably reflect true intracellular Mg variations.

In erythrocytes, other phenomena may hamper the interpretation of MHC-associated variations. EMg concentrations decrease with cell age: high in reticulocytes, they decrease and reach a steady state in mature erythrocytes and decrease again in older cells as reviewed by Gattegno *et al.* (19). Genetically low EMg values could, therefore, be ascribed to the older age of the erythrocyte population. However, erythrocyte pyruvate kinase activity and sialic acid content, which are known to decrease with erythrocyte age, are, respectively, equal and higher in $B35^+$ male blood donors than in other subjects (19). If the lower EMg values established in $B35^+$ individuals were due to the older mean age of their erythrocytes, lower pyruvate kinase and sialic acid values should be observed.

The interpretation of the genetic regulation of EMg is also hindered by the fact that the total intracellular Mg level represents both bound and free Mg^{2+} contents. Although free Mg^{2+} only constitutes a small part (≅20%) of total EMg, this is the chemical form relevant for the regulation of many biological processes (20–22). Furthermore, free Mg^{2+} levels in erythrocytes are mainly controlled by a Na^+–Mg^{2+} exchanger (23, 24), while the amount of bound Mg depends on intracellular concentrations of ATP, diphosphoglycerate, and hemoglobin (25). It is, therefore, important to assess whether the genetic control of total EMg reflects the control of one of these two fractions, bound or free Mg^{2+}, or both of them. Recent preliminary experiments (26) show that free and total EMg contents are strongly correlated in humans. In addition, the low total EMg levels of HLA-$B35^+$ subjects seem to be associated with low free Mg^{2+} contents. On the other hand, healthy male blood donors with low total EMg exhibit a high activity of the Na^+–Mg^{2+} exchanger and vice versa (27). These findings suggest that genetic factors regulating intracellular Mg might primarily control the number of transport units and/or the turnover rate of Mg^{2+} transloca-

tion by the Na^+–Mg^{2+} exchanger mentioned above. The genetic modulation of the activity of this exchanger would, consequently, affect the intracellular free Mg^{2+} level and thus the total cell Mg content, since both fractions are highly correlated. This hypothesis does not exclude the fact that intracellular Mg genetic control may act via other Mg transport systems.

In conclusion, extracellular and intracellular Mg levels appear to be regulated by a genetic system involving MHC-associated and non-MHC genes. This implies a genetic polymorphism of molecules playing a major role in Mg transport. One of these molecules could be the Na^+–Mg^{2+} exchanger described in erythrocytes (23, 24). Why is there an association between Mg transport and MHC genes? The generally accepted view of "squatter" genes intermingled by chance with "true" MHC genes coding for class I and II antigens may be an oversimplification of the problem. Let us recall that iron metabolism is partly affected by squatter MHC genes such as, for instance, hematochromatosis and ferritin genes (28). There is also some evidence that splenic zinc concentrations are H-2 dependent (29). Furthermore, steroid metabolism, which is involved in the regulation of metallic elements, is in many respects associated with the MHC (30–32). Moreover, steroids as well as magnesium (33, 34), zinc (35, 36), and iron (37, 38) play an important role in immunomodulation. The presence of genes regulating their metabolism among other MHC genes may therefore be more than nature's fantasy and contribute to the general regulation of the immune system. Two lines of mice, presently under selection for high and low EMg concentrations (39), should provide, in the near future, useful models to investigate this question. This selective breeding has been performed so far for 12 generations. Besides large differences in EMg level, the two selected lines exhibit significant differences for plasma and kidney Mg contents (unpublished data).

Meanwhile, recently published data (40) suggest that among other mouse and rat strains, those having lower blood Mg values are also those characterized by a higher humoral immune response and higher sensitivity to stress. Similarly, $B35^+$ individuals exhibit a higher titer of antibody after anti-influenza vaccination (15) and seem to be more frequently found among stress-sensitive type A behavior subjects (40). Mg-controlling genetic factors may also bring new light to some of the well-known but still little understood associations between HLA and disease susceptibility (8).

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