I-E-LINKED CONTROL OF SPONTANEOUS RHEUMATOID FACTOR PRODUCTION IN NORMAL MICE

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Rheumatoid factors (RF)[1] are defined as autoantibodies binding to the Fc region of IgG molecules, and were first found in the serum of rheumatoid arthritis patients (1, 2). Increased serum levels of RF are also detected in other autoimmune diseases in mouse and man (3–5), and are often produced as monoclonal macrogammaglobulinemias (6). Similar antibody reactivities may also be detected, however, in serum from apparently healthy individuals, and they are systematically produced in secondary, T cell–dependent antibody responses (7–11) or after polyclonal, mitogenic stimulation of B cells (12–14). Several hypotheses have been advanced to explain RF participation in joint lesion as well as their physiological interest as antibodies enhancing immune responses or antigen clearance (15).

Van Snick et al. (16–18) have described the "spontaneous" production of high titers of anti-IgG2a RF in the 129/Sv mouse strain and established the IgH-linked control of this phenotype. It was noted, in those studies, that at least one other locus participates in the control of this RF specificity and serum levels. While controlling for idiotypic binding in the normal serum of different H-2-congenic strains, we observed marked differences at the levels of IgM anti-IgG2a antibodies. Given the association between rheumatoid arthritis and HL-A DR alleles in man, and concerned with the T cell–dependent selection of antibody repertoires, particularly of those naturally produced in normal animals, we have now formally investigated the role of H-2 genes in the production of this specificity. The results presented herein demonstrate that the levels of IgM anti-IgG2a in several mouse strains are essentially controlled by genes linked to IgH and to MHC, particularly the I-E region.

Materials and Methods

Mice. C3H/HeJ, C57BL/6, B6.H-2K, C3H.SW, B10.A(4R), B10.MBr, B10.S, B10.HTT, and DBA/2 mice were bred at the Pasteur Institute. B10.Br, B10.D2, and B10.A(5R) mice were purchased from OLAC (Bicester, England), and C57BR was from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 E^d_α transgenic mice were a kind gift from Dr. Kishimoto (Osaka University, Japan) and were bred at the Pasteur Institute.

Antibodies. The myeloma proteins MOPC 173 and UPC-10 (both IgG2a, κ) were purified

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[1] *Abbreviation used in this paper:* RF, rheumatoid factor.

from ascitic fluids in a protein A-Sepharose column. The hybridoma F23.1 (IgG2a, κ) (19) was purified by the same procedure from culture supernatants.

Detection and Quantification of IgM Anti-IgG2a RF. Polyvinyl plates were coated with the different monoclonal IgG2a, or a mixture of them (10 μg/ml) in 50 mM phosphate buffer overnight at 4°C. The plates were then incubated at room temperature for 1 h with PBS containing 1% gelatin, for 2 h with serial dilutions of individual mouse sera, and for 1 h with peroxydase-labeled goat anti-mouse μ chain antibodies (Southern Biotechnologies, Birmingham, AL), with extensive washes between each step. The enzyme reaction was developed for 20 min, using *o*-phenylendiamine (Sigma Chemical Co., St. Louis, MO) as a substrate, and quantified photometrically in a Multiscan (Flow Laboratories, Irvine, UK) at a 450-nm wavelength. In every plate, a monoclonal IgM anti-IgG2a was titrated and used as standard. All the data are expressed as equivalents (in μg/ml) of the mAb.

Typing of IgCH Allotype. The IgM and IgG2a allotypes were identified by a solid phase assay in ELISA using the following antiallotypic mAbs: anti-Ig6^b MB.86 (20); anti-Ig1^b G12-47.30 (a kind gift from Dr. P. Vieira, Cologne University, FRG); anti-Ig6^a RS3.1 (21); and anti-Ig1^a Ig1a (8.3) (22).

Polyvinyl plates were coated with the different antiallotypic mAbs at 10 μg/ml. After saturation and incubation with serial dilutions of individual sera, they were incubated with the same biotin-labeled mAbs used for the coating, followed by a 1-h incubation with peroxydase-conjugated avidin (Milles, Bethesda, MD). All other steps in the ELISA technique were identical to those above.

Typing of H-2. The H-2 haplotype was typed in a solid phase RIA with fixed white blood cells. Briefly, mice were bled in heparinized tubes and the white cells enriched by centrifugation in a Ficoll. A serial titration of the enriched population was fixed with glutaraldehyde to polystyrene plates, and incubated with ^{125}I-labeled anti-I-E$^{d/k}$ (14.4.4) (23), or anti-I-A^b (25.9.17S) (24) antibodies. After extensive washing, the radioactivity was measured in a gamma counter. Alternatively, white blood cells or spleen cells were incubated with the same antibodies labeled with fluorochromes and analyzed in a FACScan (Becton Dickinson & Co., Mountain View, CA).

Statistical Analysis. Typing of individual animals for IgM anti-IgG2a RF expression, in the progenies of the crosses analyzed, was done as follows. All animals in the cross, irrespective of the expression of other segregating markers, were distributed in classes defined by the concentration of IgM anti-IgG2a serum antibodies. In cases where this trait is segregating, two roughly gaussian populations become evident in the distribution, with a cutting point at ~3–5 μg/ml equivalents, allowing for the classification of high and low expression, regardless of other groupings within each of these populations (see below).

Results

Serum Levels of IgM Anti-IgG2a RF in C3H/HeJ, C57BL/6, respective F_1 Hybrids, and H-2 Congenic Strains. In the course of unrelated experiments, we noted marked differences in the titers of IgM antibodies binding to ELISA plates coated with IgG2a preparations that could be detected in the sera from various mouse strains. We have ascertained: (*a*) the strain dependence of this phenomenon, by the comparable levels detected in many individual sera within a given strain; and (*b*) the RF specificity of such IgMs, first, by finding comparative binding of the same sera to three different IgG2a mAbs or mixtures thereof, and second, by the complete removal of serum IgM binding to all three IgG2a mAbs by immunoabsorption of the serum on a Sepharose column coupled with one of them only (data not shown). We proceeded, therefore, to quantitate such RF in the serum of a variety of mouse strains, and chose to analyze in detail C3H/HeJ (C3H) and C57BL/6 (B6) as prototypes of high and low expressors, respectively (Fig. 1). Not only were the differences between these

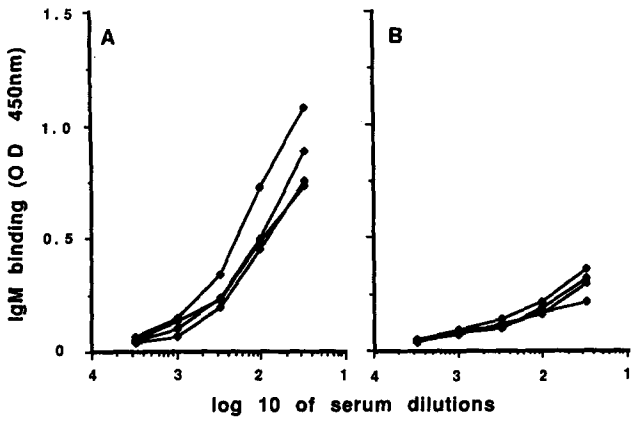

FIGURE 1. C3H/HeJ (*A*) or C57BL/6 (*B*) individual sera were titrated into plates coated with a mixture of MOPC-173, UPC-10, and F23.1 antibodies. Bound IgM was detected with μ chain-specific, peroxydase-labeled antibodies. Each point represents the mean OD of duplicate determinations.

two strains readily detectable, but also, we had available the appropriate H-2 congenic and RI lines, as well as intra-H-2 recombinants in b/k haplotypes.

The finding of high RF titers in normal C3H mice is reminiscent of Van Snick's observations (25). We have also confirmed the age dependence of the serum titers and the prevalence of IgM RF in younger animals, in contrast to the predominance of IgA RF in animals older than 4–6 mo. Because background genes appear to have a determinant effect on the total levels of IgA production, very different in C3H and B6 strains, we will only consider here IgM RF in relatively young animals (8–16 wk), where levels of total serum IgM are comparable, although IgA RF were also quantitated in all experiments. Even if RF titers continue to increase with age, amplifying the strain differences, these are already sufficiently marked at that age to allow for unambiguous typing.

The serum IgM RF concentrations might be in part controlled by MHC-linked genes, as suggested by the study of MHC-congenic animals to the prototype strains. As shown in Fig. 2, B6.H-2K mice type as C3H, and C3H.SW type as B6. Fig. 2 also shows that F_1 hybrids between high and low expressors of RF are all low expressors, demonstrating that in these cases the trait low expressor phenotype is dominant, as it has already been shown in several independent progenies of 129/Sv strain crosses (17, 18). In addition, (B6.H-2K × C3H)F_1 hybrids were also found to be low expressed. These results are already suggestive that H-2-linked and unlinked genes both contribute to RF phenotype.

Segregation Analyses of Backcross Progenies between High and Low IgM Anti-IgG2A RF Expressors: IgH- and H-2-linked Control. The suggestion of MHC-linked control provided by the congenic strains was evaluated by conventional genetic analyses in progenies of backcrosses between the prototype strains, segregating for H-2, IgH-allotype, and RF phenotype. Backcrossing of (C3H × B6)F_1 mice to high expressor C3H mice resulted in progenies that segregated for serum RF concentrations, in a pattern compatible with a genetic control by one or two unlinked loci (numbers of low and high expressors being 26 and 41, respectively).

Typing of these progenies for IgH haplotypes shows clear linkage of RF levels to

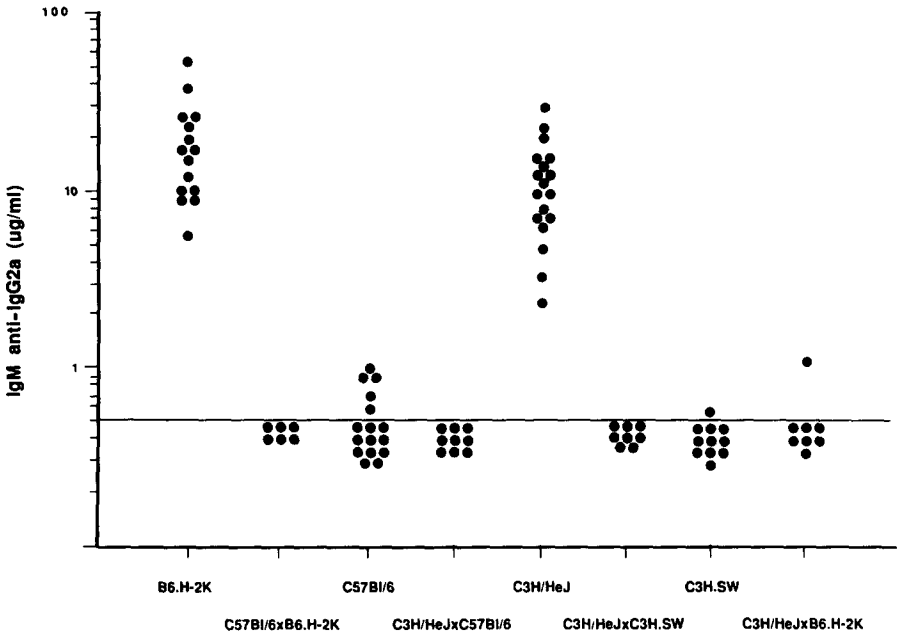

FIGURE 2. Different H-2-congenic mouse strains and F_1 hybrids. Each point corresponds to an individual mouse. Mice were tested between 8 and 14 wk of age.

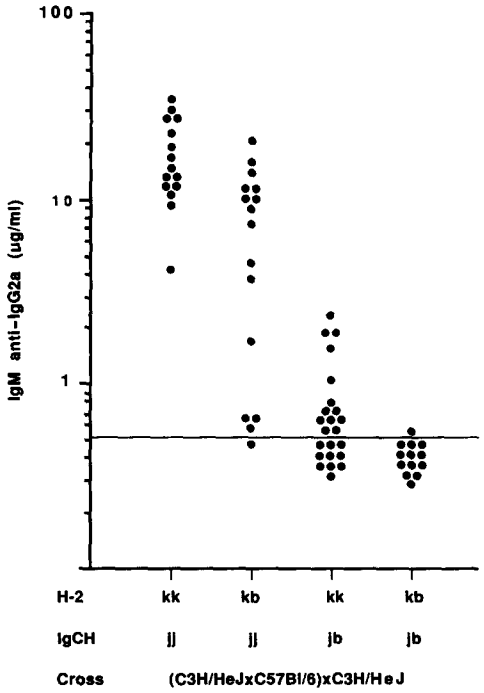

FIGURE 3. (C3H/HeJ × C57BL/6)F_1 × C3H/HeJ mice distributed as to their H-2 and IgH haplotypes. Each point corresponds to an individual mouse and represents the mean of six different determinations using three different IgG2a antibodies (see Materials and Methods). Mice were tested at 12 wk of age.

the IgH locus. Thus, all high expressors are IgH^j homozygous (Fig. 3 and Table I). Furthermore, a closer scrutiny of the quantitative levels of RF expression, together with H-2 typing of these mice, reveals that high and low expressors are both clustered in two subgroups showing a precise linkage to H-2: H-2K homozygocity determines higher levels of expression in both IgH allotype groups (Fig. 3 and Table II). It is also interesting to note that H-2 heterozygous backcrosses to C3H are high RF expressed in contrast to F_1 animals. This indicates that there are assortments of C3H and B6 genomes that are compatible with high RF expression even in H-2 heterozygous animals.

Fig. 3 also shows that in animals carrying IgH or H-2 haplotype combinations that are not those of prototype high or low expressors, RF seem to fall into, yet, other clusterings. This could suggest the existence of a third locus controlling quantitatively the levels of RF expression. With too few animals analyzed here for this contention, we dare to advance this suggestion because of published data by Van Snick (17) concerning F_2 and backcrosses of 129/Sv and C57BL/6 strains. In such crosses, where the segregating H-2 haplotypes could be expected to have minor or no effects, clustering of animals within IgH-defined groups was also observed. More specific information concerning this issue can be obtained by the analysis of available F_2 recombinant inbred strains between C3H and B6, as well as their crosses to either parental strain, which might provide a definite conclusion.

TABLE I

IgCH Allotype-linked Gene Control of RF Expression in F_1(C3H × B6) × C3H Progenies

IgH haplotype	Number of mice	Number of mice with >3 μg/ml IgM anti-IgG2a	t*	*p*
jj	32	26		
jb	35	0	50.1	$<5 \times 10^{-4}$

Animals with >3 μg/ml IgM anti-IgG2a RF were considered as high expressors (see Materials and Methods).

* Considering all the animals. If only high expressor animals are considered, the differences are still significative ($t = 2.47$ $p < 0.05$).

TABLE II

H-2-linked Gene Control of the RF levels in F_1(C3H × B6) × C3H Progenies Homozygous for $IgCH^j$ Haplotype

H-2 haplotype	Number of mice	Number of mice with >3 μg/ml IgM anti-IgG2a	IgM anti-IgG2a titers + *SD*	t*	*p*
kk	15	15	17.7 + 8.8	3.79	<0.01
kb	16	11	7.5 + 6		

Animals with >3 μg/ml IgM anti-IgG2a RF were considered as high expressors (see Materials and Methods).

* Considering all the animals. If only high expressor animals are considered, the differences are still significative ($t = 2.47$ $p < 0.05$).

Given the apparent complexity of this genetic control, H-2 linkage could be best addressed in backcrosses where all the other loci are fixed, namely between two H-2-congenic strains. This experiment would also give an internal control for the discriminatory ranges of the assay detecting RF. Fig. 4 shows the results of such crosses between (C3H/HeJ × C3H.SW)F_1 back to C3H/HeJ, where all but one H-2^k homozygous mouse have high levels of anti-IgG2a RF, while, again, all but one H-$2^{k/b}$ heterozygous mouse are low expressor. Even including those two exceptional animals, which might reflect an experimental error, the linkage between high RF expression and H-2 in these backcrosses is statistically significant ($p < 0.01$).

I-E Control of IgM Anti-IgG2a RF Production. We next set out to investigate which subregion of the H-2 complex is involved in the regulation of the serum levels of IgM anti-IgG2a RF. Table III shows the results obtained with different intra-H-2-recombinant mice, congenic to C57BL.10. As can be seen, mice of the B10.A(4R) strain are low expressors (like B10 and B6), while B10.Br, B10.MBR, and B10.A(5R) are high expressors. These data suggest that the expression of I-E, or of a gene closely linked to it, is sufficient and necessary for the expression of high levels of IgM anti-IgG2a RF in the C57BL background. The same conclusion is supported by the analysis of intra-H-2-recombinant inbred strains between H-2^s (I-E nonexpressor) and H-2^k (I-E expressor) haplotypes. As can be also seen in Table III, B10.S (I-E^-) behaves as a low expressor, while B10.HTT (I-E^+) appears as a high expressor. Furthermore, another I-E^+ H-2-congenic strain with B10, namely B10.D2 carrying the d haplotype, also types as high RF expressor.

To ascertain whether the serum levels of IgM anti-IgG2a are directly controlled by the expression of the I-E molecule, or by some other genes closely linked to the I-E region, we analyzed C57BL/6 mice carrying the I-E^d_α transgene (26). As shown in Fig. 5, these mice express increasing levels of seric IgM anti-IgG2a with age in contrast to their nontransgenic littermates, which express undetectable levels of anti-IgG2a IgM. These results demonstrate that the expression of the I-E molecule is

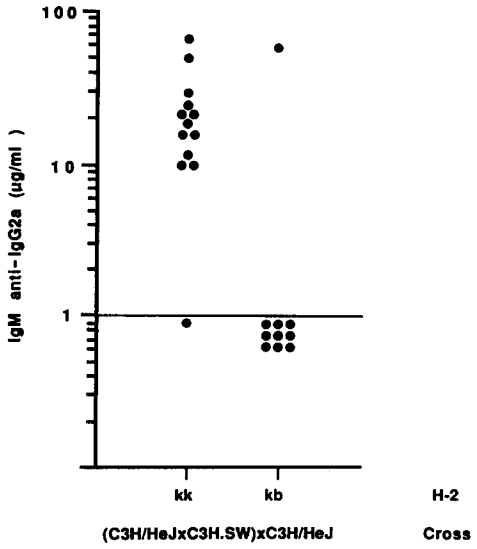

FIGURE 4. Expression of IgM anti-IgG2a RF in crosses between (C3H/HeJ × C3H.SW)F_1 × C3H/HeJ mice distributed as to their H-2 haplotypes. Each point corresponds to an individual mouse tested at 16 wk of age.

TABLE III

RF Expression in Different Intra-H-2-recombinant Strains of Mice

Strain	H-2 K	I-A	I-A	I-E	I-E	D	Animals with >3 μg/ml IgM anti-IgG2a	IgM anti-IgG2a ± *SE (μg/ml)*
B10.Cr	b	b	b	b	-	b	0/5	<1
B10.Br	k	k	k	k	k	k	8/10	7.2 ± 1.46
B10.MBr	b	k	k	k	k	q	4/4	9 ± 1.3
B10.A(4R)	k	k	k	k	-	b	0/5	<1
B10.A(5R)	b	b	b	b	k	k	-*	9*
B10.S	s	s	s	s	-	s	0/5	<1
B10.HTT	s	s	s	s	k	k	5/5	18 ± 3.6
B10.D2	d	d	d	d	d	d	6/6	7.6 ± 0.91

* Only a pooled serum from five animals was tested.

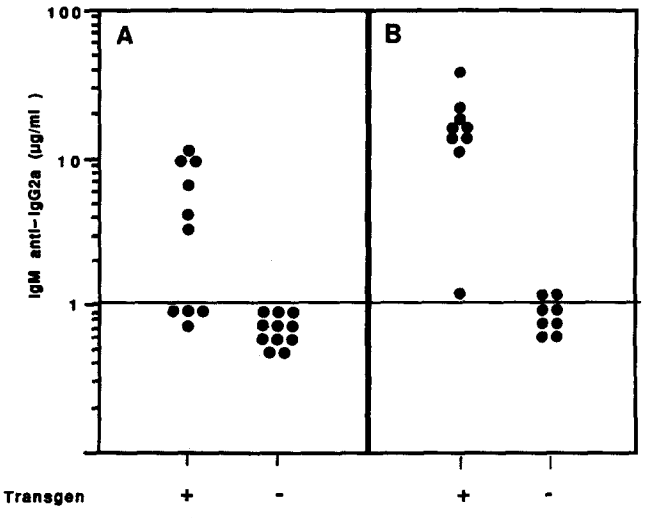

FIGURE 5. Expression of IgM anti-IgG2a RF in C57BL/6 (E^d_α) transgenic mice and their littermates at 7 wk (*A*) or 14 wk (*B*) of age. Each point corresponds to an individual mouse.

sufficient and necessary for the production of high levels of IgM anti-IgG2a RF in the C57BL/6 strain.

Discussion

The production of RF able to bind autologous IgG in certain specific pathogen-free mouse colonies has been shown to be strain specific in quantitative and qualitative terms, and appears to be controlled by both genetic and environmental factors. Those RF belong to the IgM class in young animals and switch, progressively with age, to IgA (16, 25). By crossing 129/Sv mice, as a prototype of high RF expressors, with different nonexpressor F_1 hybrid mice, Van Snick et al. (17, 18) have shown the dominance of the low RF phenotype and a linkage to the IgCH allotype or to MHC genes.

We have now analyzed the genetic control of spontaneous RF production using C3H as a high expressor prototype strain. Our data confirm a major role for IgH

allotype-linked genes in the RF expressor phenotype, show a clear control by the H-2 complex and, more precisely, map it to the I-E locus. Considering this multiple genetic control of RF production, several important points should be discussed.

First, we note the finding that F_1 hybrids between high and low RF expressors type as low expressors, that is, the low RF phenotype is dominant. This is true for all six F_1 hybrids that we have tested as well as for all crosses previously described by Van Snick (17, 18), using the 129/Sv strain as high expressor and a number of low expressor strains. F_1 low expressor phenotype is then a major characteristic of the genetic control of RF production. Thus, heterozygocytes of H-2 only, e.g., (B6 × B6.H-2K)F_1 or (C3H × C.SW)F_1, of non-H-2 genes only, e.g., (B6.H-2K × C3H)F_1 or (BALB/c × B6)F_1 (not shown) are sufficient to suppress RF expression. This, however, does not seem to reflect suppressive mechanisms of the type controlled by conventional *Is* genes. Thus, H-$2^{k \times b}$ heterozygous can actually be high expressors, as shown here with (C3H × B6)F_1 × C3H backcrosses, or by crossing (C3H × B6)R_I lines carrying H-2^k with B6 (unpublished observations). It would seem, therefore, that if at all resulting from suppressive mechanisms (see below), low RF expression would be the consequence of interactions between the products of H-2 alleles and those of other non-MHC polymorphic genes.

Second, when low expressor F_1 hybrids are backcrossed to the high expressor parental strain, a clear linkage to the IgH locus is shown. In our data, this is even more striking because both IgH haplotypes studied appear to be equally competent when placed in the appropriate background (e.g., the j allotype in C3H and the b allotype in B6.H-2K). This suggests that the control of RF production by IgH-linked genes does not reflect the absence or presence of a particular set of V_H genes, but rather, the regulation of the expression of those genes, more particularly the selection of the B cells producing RF. Alternatively, since we deal here with the production of autoantibodies, IgH linkage could reflect polymorphism of the autoantigen (IgG_{2a} C regions).

The role of H-2-linked genes is more complex to discuss. The analysis of intra-H-2-recombinant strains in the B10 background and of the C57BL/6-transgenic mice carrying the I-E_α gene demonstrates that the expression of the I-E molecule is sufficient and necessary for the high RF phenotype in that background. This linkage supports the notion that specific T cells are required in the spontaneous production of RF (27), as it has been shown in the course of secondary responses to protein antigens (7–10), and suggests that low RF production in B6 is not the result of suppressive mechanisms. I-E expression, regardless of its "fine specificity," seems here to be essential, since all four E alleles tested (of the k, b, s, and d haplotypes) ensure high RF expression. The situation appears quite different, however, from the *Ir* gene effects (helper or suppressor), be those resulting from either positive or negative selection of T cell repertoires. Thus, if I-E were functioning in positive selection of some T cell specificities, in agreement with the "responder" phenotype of the parental strains, we would expect a "high responder" phenotype in F_1 hybrid animals (27), and this is not the case. Conversely, if I-E expression would result in clonal deletion of "suppressive" T cells, we would again expect dominant high RF expression. Finally, if I-E functions by positive selection of suppressors cells, as suggested by the F_1 phenotype, we would expect for the parental strains an expression of RF

that is the opposite of what is observed. One possible explanation invokes quantitative differences in the cellular density of I-E expression, which in a (k × b)F_1 should only be half of the homozygous or transgenic animals. Another possibility could postulate the involvement of other MHC (but not I-E) genes in a complex control of this phenotype, which, not unlikely, may involve various clones of cooperative and regulatory cells.

These considerations on H-2 controls should nowadays be taken together with putative polymorphisms in genes encoding (self) proteins that may associate with MHC molecules and determine negative or positive selection of T cell repertoires (28–33). As seen above, the structural antigen itself (C regions of IgG_{2a}) may be one of these. Any other background gene, however, could result in similar effects. Finally, TCR genes are likely to be implicated in the regulation of this phenotype (27), which is then the result of complex selective processes that involve the products of several loci. Alleles at each of these loci may be either permissive or "suppressive" in the context of different "background" genetic constitution. It is, therefore, inappropriate to conclude on the participation or noninvolvement of a particular locus (e.g., Mls-1, TCR-β, etc.) by the typing of independent strains, although we have much data already available.

In summary, both the IgH-linked and I-E gene, which undoubtedly control RF expression, provide for high or low expression phenotypes depending upon homo- or heterozygocity, the allelic context of the other locus, and other background genes. This complex genetic control suggests that the spontaneous production of high RF levels requires multiple molecular and cellular interactions between different lymphocyte subsets, the repertoires of which may have had their selection determined by those loci. Given the close association between allelic forms of DR and rheumatoid arthritis in humans (34), our results indicate that further efforts in the characterization of those mechanisms and interactions are urgent.

Summary

The concentration of serum IgM molecules binding to IgG2a (rheumatoid factor [RF]) in solid phase assays is 10–100-fold higher in normal, unmanipulated C3H/HeJ ($H\text{-}2^k$) than in C57BL/6 ($H\text{-}2^b$) mice. Analysis of MHC-congenic mice with the prototype strains show that C3H SW ($H\text{-}2^b$) are low, and B6.$H\text{-}2^k$ are high RF expressor strains, respectively. Furthermore, segregation of RF phenotypes in progenies from backcrosses to C3H/HeJ of (C3H/HeJ × C57BL/6)F_1 hybrid mice shows MHC- and IgH-linked controls. RF phenotypes also segregate as if they are MHC linked in crosses between H-2-congenic strains (C3H/HeJ and C3H.SW). The study of intra-H-2 (k/b and k/s) recombinant mice suggested that RF phenotype control is linked to the I-E region. This was confirmed by the typing of C57BL/6 mice expressing a transgenic Eα chain, and thus, I-E^+, which, in contrast to nontransgenic littermates, are high expressors of RF.

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