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Mitochondrial modulation of maternally transmitted antigen: Analysis of cell hybrids

(murine histocompatibility complex/mtDNA/rhodamine 6G)

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ABSTRACT **Maternally transmitted antigen (Mta) is a murine cell surface class I-like antigen that is defined by specific cytotoxic lymphocyte reactivity. Mta is unique in that its expression requires cooperation between genetic elements both in the *Qa/Tla* region of chromosome 17 and in the cytoplasm. In view of the known cytoplasmic, and thus maternal, inheritance of mitochondria, we have directly assessed their potential involvement in Mta expression. The mitochondria-specific lethal dye rhodamine 6G (R6G) was used to control the input of mitochondria into cell hybrids. The parental lines, one of BALB/c and one of NZB origin, were known to differ in Mta and mtDNA phenotype. Our data show that most control BALB/c–NZB hybrids expressed the BALB/c Mta phenotype and likewise contained only BALB/c-type mtDNA. The NZB Mta phenotype was not coexpressed in the control hybrids. However, when the mitochondrial contribution from BALB/c was prevented by R6G treatment, the majority of the resultant hybrids expressed only the NZB Mta type and likewise contained only NZB mtDNA. The exceptional R6G-treated hybrids that continued to express the BALB/c Mta phenotype likewise contained only BALB/c mtDNA. Thus, in every case the mtDNA phenotype correlated with the Mta phenotype of the cells. Together, the data support the remarkable conclusion that mitochondria modulate the phenotypic expression of a cell surface molecule.**

Maternally transmitted antigen (Mta) is a recently identified murine cell surface antigen that serves as a target for H-2 nonrestricted killing by cytotoxic T lymphocytes (CTL) (1, 2). Mta has properties suggesting a structural homology with class I molecules of the major histocompatibility complex, including probable association with β_2-microglobulin (β_2m) on the cell surface (3). Unlike any other known cell surface molecules, Mta expression is determined by maternal phenotype. This phenotype is not modified by foster mothering, embryo transfer, or transfer of bone marrow cells to lethally irradiated mice (4, 5). Thus, it is unlikely that a conventional infectious agent specifies production or alters recognition of this antigen. Inheritance of Mta was initially thought to be strictly non-mendelian and thus transmitted through the cytoplasm of the ovum (4). The cytoplasmic element was shown to be stable through 11 generations of backcrossing (5).

It is now known that expression of Mta requires cooperation between a maternally transmitted genetic element, *Mtf*, and a 17th chromosome gene, *Hmt* (3). Fisher Lindahl has found that *Hmt* maps to the *Tla* region of the *H-2* complex. All mice of the common laboratory strains are similar with respect to *Hmt* with the exception of strains derived from the *Mus musculus castaneus* subspecies in which the *Hmt* allele appears to be silent. Several alleles of the cytoplasmic element *Mtf* have been hypothesized, each controlling expression of a distinct Mta phenotype (3). Identification of the genetic element represented by *Mtf* is vital to understanding how a cytoplasmic factor interacts with a chromosomal gene product to produce a cell surface antigen. Because mitochondria are cytoplasmically, and thus maternally, inherited (6), the relationship between mitochondria and Mta expression has been investigated (7–9). Restriction endonuclease analysis has shown that most old inbred strains of mice share the same type of mtDNA (10–12). They are likewise identical in Mta expression (1). Recent studies in our laboratory (7), and that of Ferris *et al.* (8), revealed unique mtDNA restriction enzyme patterns in mice expressing Mta phenotypes different from that of the old inbred strains. These studies could not rule out coincidental inheritance of *Mtf* and a particular mtDNA type due to a shared maternal mode of transmission. Therefore, we assessed directly the potential involvement of mitochondria in Mta expression (9). The mitochondria-specific lethal dye (13, 14) rhodamine 6G (R6G) was used to control the input of viable mitochondria from one fusion partner into cell hybrids. The splenocyte and plasmacytoma fusion partners chosen were known to differ in Mta phenotype and mtDNA type (7). Untreated control hybrids expressed the plasmacytoma Mta phenotype. However, when the plasmacytoma partner was pretreated with R6G and, therefore, could not contribute viable mitochondria, the resultant hybrids no longer expressed the plasmacytoma Mta phenotype (9). Thus, our previous studies suggested a causal relationship between mitochondrial origin and Mta expression. However, it was important to prove that R6G treatment had the intended effect of preventing the transmission of BALB/c mitochondria into the hybrid cells and that the Mta phenotype correlated directly with the mitochondrial type.

In this investigation, we have further analyzed BALB/c–NZB somatic cell hybrids for mtDNA restriction endonuclease fragment length polymorphism and with cloned CTL lines that recognize Mta of either the BALB/c (Mta^a) or NZB (Mta^b) type. By demonstrating that loss of the Mta^a phenotype in R6G-treated hybrids is associated with acquisition of Mta^b- and NZB-type mtDNA our results considerably strengthen and extend the interpretation that mitochondria are the cytoplasmic elements involved in controlling the phenotypic expression of Mta.

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Abbreviations: Mta, maternally transmitted antigen; R6G, rhodamine 6G; CTL, cytotoxic T lymphocyte(s).

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MATERIALS AND METHODS

Mice and Cell Lines. NZB/BlNJ mice were obtained from The Jackson Laboratory. BALB/cCrgl mice were obtained from the Cell Biology Department, Baylor College of Medicine. (BALB/cCrgl × NZB/BlNJ) F_1 and (NZB/BlNJ × BALB/cCrgl) F_1 mice were bred in our animal facilities. The BALB/c-derived, 8-azaguanine-resistant plasmacytoma cell line P3x63/Ag8.653 (15) was obtained from D. Via, Baylor College of Medicine. The parental order of crosses is designated (female × male) F_1.

Hybrid Production. The fusion protocol has been described (9). Briefly, x63/Ag8.653 cells either were treated for 48 hr with R6G (Kodak), at 1 μg/ml in Dulbecco's modified Eagle medium, or left untreated. They were then fused with normal NZB/BlNJ splenocytes by using polyethylene glycol (M. A. Bioproducts, Walkersville, MD). Hybrid clones were selected in hypoxanthine/aminopterin/thymidine medium, expanded, and assayed for Mta expression. The clones described here are designated with the prefix "C" before control hybrids and "R6G" before treated hybrids.

Generation of Long-Term CTL Lines. The development of cloned CTL lines has been described (16). The Mta.1-specific CTL lines 5F3 and 91D1 were established and maintained by repeated stimulation of Mta^b (NZB × B10.D2) F_1 splenocytes with irradiated Mta^a BALB/c spleen cells and interleukin 2. The Mta.2-specific CTL lines 6C10 and 4A6 were similarly established and maintained, but with repeated stimulation of Mta^a (B10.BR × NZB) F_1 splenocytes with irradiated Mta^b NZB spleen cells. CTL assays were performed as described (9). Tests to rule out the possibility that the CTL lines recognized antigens other than Mta have been described (9, 29). The killing pattern, dependent solely on the parental order of the crosses, establishes both anti-Mta.1 CTL and anti-Mta.2 CTL as specific for a maternally transmitted antigen.

All cells and mice used in this study possess the 17th chromosome coding allele Hmt^a (3). The alleles of *Hmt* are designated by the superscript "*a*" or "*b*". The presumed alleles of *Mtf* are designated by Greek letters (3). Of the cells and mice used, only the NZB/BlNJ strain is Mtf^β; all others are Mtf^α. The alternative forms of the Mta molecule itself are designated by the superscript "a" or "b", to correspond to the associated *Mtf* allele. Finally, the determinants on the Mta molecule recognized by the CTL lines are given the designation Mta.1 and Mta.2, to correspond to the "a" and "b" forms of the molecule.

mtDNA Preparation. The hybrid lines were expanded to yield 1 ml of packed cells. Mitochondria were isolated by the sucrose gradient purification method of Bogenhagen and Clayton (17). mtDNA was purified by using the alkaline lysis method of Portnoy *et al.* (18). The DNA preparation was extracted twice with phenol/chloroform, extracted three times with chloroform/isoamyl alcohol (24:1), extracted once with ether, and reprecipitated by using 0.5 vol of 7.5 M ammonium acetate and 2 vol of ethanol.

Restriction Endonuclease Analysis. The purified DNA was digested overnight by using *Hin*fI (New England Biolabs) as recommended by the supplier. The digested DNA was end-labeled with [^{32}P]dATP, electrophoresed on 5% polyacrylamide gels, and autoradiographed, as described by Brown (19).

RESULTS

Cell hybrids were constructed to test the hypothesis that mitochondria control expression of Mta (9). The contribution of mitochondria from a BALB/c plasmacytoma (x63/Ag-8.653) into subsequent hybrids with NZB splenocytes was ablated by R6G treatment. R6G is a highly lipophilic dye that binds irreversibly to mitochondrial inner and outer membranes, blocking oxidative phosphorylation (13). Fusion with untreated splenocytes rescued the lethally exposed plasmacytoma partner with little effect on the generation of hybrids (9, 14). R6G pretreatment did not influence expression of the nuclear-encoded Qa-1.2 cell surface antigen (9).

Our experimental strategy was based on the following lines of reasoning. (*i*) Control hybrids between x63/Ag8.653 of BALB/c origin and NZB/BlNJ splenocytes would be Hmt^a but may have either or both cytoplasmic factors, Mtf^α and Mtf^β. (*ii*) The Mta phenotype of these hybrids would be dependent on the potential for dominance or coexpression if, indeed, both factors are retained in the hybrid cytoplasm. (*iii*) R6G-treated hybrids would also be Hmt^a and would have cytoplasmic contributions from both parents but would have viable mitochondria from the NZB parent only. If mitochondria influence Mta expression, the phenotype of these hybrids should be Mta^b because only Mtf^β would be present. (*iv*) Finally, because it is possible to distinguish the mtDNA from murine strains that express Mta^b from the mtDNA of strains expressing Mta^a (7, 8, 10), the hybrids could be tested for mtDNA type. If Mta expression is controlled by mtDNA, the mtDNA type should correlate with the Mta determinant(s) expressed.

Phenotyping of Hybrids with Mta-Specific CTL. Initial typing of the x63/Ag8.653–NZB hybrids showed that of the 10 clones tested from the untreated fusion, all were $Mta.1^+$. In contrast, 76% (23/30) of the hybrids derived from R6G-treated x63/Ag8.653 cells failed to express Mta.1 (9). After the initial typing studies were completed, we employed reciprocal immunization protocols to develop CTL lines specific for determinants of Mta^b carried by NZB mice and designated Mta.2 (29). A small sample of the hybrid lines constructed was retested for expression of Mta.2 and for mtDNA phenotype. This sample included typical and atypical members of both control and treated hybrid lines.

Of four hybrids typed from the control fusion, three (C1, C2, C3) expressed only Mta.1 and were $Mta.2^-$. However, one control hybrid, C4, was susceptible to killing by both Mta.1- and Mta.2-specific CTL (Table 1). Cloning of this hybrid revealed the presence of two populations, one of which expressed only Mta.1 and the other only Mta.2. In the control fusions, cytoplasmic elements from both parent cells

Table 1. Mta phenotyping of control and R6G-pretreated hybrids

Target cells	CTL line and specificity			
	5F3 (Mta.1)	91D1 (Mta.1)	6C10 (Mta.2)	4A6 (Mta.2)
Con A blast splenocytes				
(NZB × BALB/c) F_1	−3	0	56	47
(BALB/c × NZB) F_1	57	49	3	2
Control hybrid				
C1	41	50	−1	−1
C2	18	51	1	1
C3	21	37	0	2
C4	52		49	
Treated hybrid				
R6G1	1	−1	38	25
R6G2	1	0	55	42
R6G3	3	0	40	38
R6G4	31	56	0	4

Hybrid cell lines were assayed for susceptibility to lysis by the indicated CTL lines. The data represent the mean % net ^{51}Cr release values of two or three experiments. The effector:target ratio was 3:1 for all experiments and the spontaneous release varied from 11% to 32% of the maximal release. The standard error of the % net ^{51}Cr release was <3% for each of triplicate determinations.

contributed to the hybrid cytoplasm and yet coexpression of Mta.1 and Mta.2 was not observed.

Of four hybrids derived from the R6G-treated parent, all but one did not express Mta.1 (Table 1). However, recognition by Mta.2-specific CTL revealed that these hybrids did express the determinant derived from the NZB parent. One of the hybrids derived from the treated fusion R6G4 was among a minority that consistently expressed Mta.1. Table 1 shows that Mta.2 was not coexpressed by this hybrid.

Restriction Endonuclease Analysis. C1 was a typical hybrid from an untreated fusion in that it was Mta.1$^+$ and Mta.2$^-$. As shown in Fig. 1, the mtDNA type of this hybrid was the same as its x63/Ag8.653 (BALB/c origin) parent with no evidence of cleavage sites present in NZB mtDNA. Hybrid R6G1 was typical of clones derived from the R6G-treated fusion in that its phenotype was Mta.1$^-$ and Mta.2$^+$. The mtDNA of this hybrid exhibited a *Hin*fI restriction enzyme pattern that is typical for NZB mice (7), with no evidence of bands representing *Hin*fI cleavage sites found in BALB/c-type mtDNA. An exception to our general finding that R6G-treated hybrids were exclusively Mta^b and displayed NZB-type mtDNA was represented by one hybrid, R6G4. Although R6G4 was derived from a treated fusion, its phenotype was consistently Mta.1. Importantly, its mtDNA was likewise of the BALB/c type. As noted above, a second anomalous finding was represented by a control hybrid, C4,

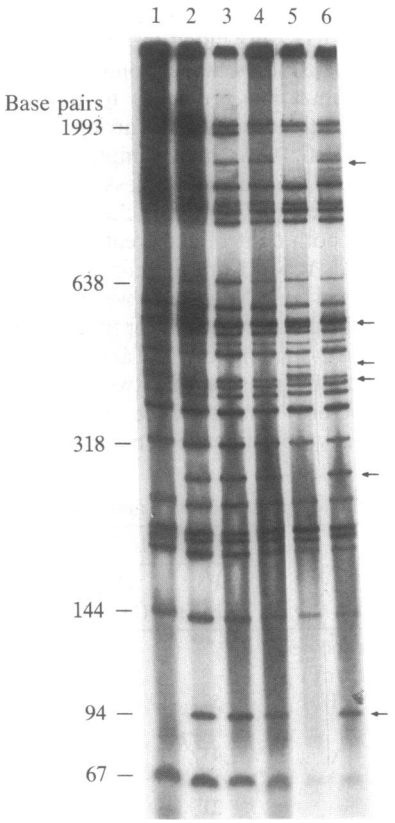

FIG. 1. Polyacrylamide gel electrophoresis of *Hin*fI digests of mtDNA samples from hybrid cells and x63/Ag8.653 (653) control. The base pair values were determined by computer search for *Hin*fI sites in the sequenced mouse L-cell mitochondrial genome (20). L cells originated from a C3H/An mouse (21), an old inbred strain sharing the same mtDNA type as of BALB/c (11). The arrows indicate places at which the patterns of BALB/c and NZB mtDNA differ. Differences in staining intensity between bands is probably a function of the labeling techniques as silver-stained gels of similar mtDNA types do not indicate a greater representation of some fragments. Lane 1, NZB/BINJ; lane 2, BALB/c; lane 3, x63/Ag8.653; lane 4, C1; lane 5, R6G1; lane 6, R6G4.

Table 2. Correspondence of Mta and mtDNA phenotypes in parental cells and hybrids

Parental cells and hybrids	Mta	Determinant	mtDNA*
BALB/c x63/Ag8.653	a	Mta.1	A
NZB splenocytes	b	Mta.2	H
Control hybrid			
C1	a	Mta.1	A
C2	a	Mta.1	A
C3	a	Mta.1	A
R6G-treated hybrid			
R6G1	b	Mta.2	H
R6G2	b	Mta.2	H
R6G3	b	Mta.2	H
R6G4	a	Mta.1	A

*Ferris *et al.* (8) used these fragment pattern-type designations for BALB and NZB mtDNA cleaved with *Hin*fI. They are used here to facilitate comparison.

which expressed both Mta determinants. Restriction endonuclease analysis of this line revealed a mixed mtDNA pattern (data not shown). However, on recloning, this line was proven to be a mixed population of Mta^a and Mta^b cells. Thus, in these and other hybrids examined, the Mta phenotype matched the mtDNA type of the hybrid cells with no evidence of coexpression, cohabitation of mitochondria, or recombination of mtDNA resulting in novel restriction endonuclease fragments. Table 2 compares the Mta and mtDNA phenotypes of the lines described. In every case, hybrids expressing the BALB/c-type Mta.1 contained only BALB/c-type mtDNA. Likewise, hybrids expressing the NZB-type Mta.2 contained only NZB-type mtDNA.

DISCUSSION

Mta is a unique cell surface molecule in that its expression is influenced by both chromosomal (*Hmt* and $\beta_2 m$) and cytoplasmic (*Mtf*) genes. Construction of hybrids between cells genetically identical with respect to *Hmt*, but differing in *Mtf*, allowed for probable identification of the source of *Mtf*. Pretreatment of one hybrid partner with a mitochondria-specific lethal dye prevented expression of the Mta phenotype of that parent in hybrid cells (9). However, the conclusion that mitochondria are thus responsible for Mta expression is greatly strengthened by the demonstration in this paper that (*i*) the hybrid cells, after ablation of mitochondria from one partner, express the phenotype of the second and (*ii*) the restriction endonuclease cleavage pattern of mtDNA isolated from each hybrid matches its Mta phenotype. It is implicit in this argument that R6G affects only mitochondria. We have considered the possibility that a hypothetical cytoplasmic genome encoding *Mtf* is associated with lipid and is thus sensitive to R6G. However, the association of a coding structure (DNA) with lipid is necessary but not sufficient for susceptibility to R6G. The only known essential feature of R6G action is the inhibition of oxidative phosphorylation, possibly by blocking the function of the ADP/ATP translocase (13). For an extramitochondrial structure to be affected by R6G, it would probably share the property of energy transduction with mitochondria. No such structures have been described. The detection of only NZB-type mtDNA in many of the treated hybrids shows that R6G treatment had the intended effect of preventing the transmission of viable BALB/c mitochondria.

When the cell hybrids described herein were initially typed, only one phenotype of Mta was known (9). Most mouse strains were considered Mta$^+$ with the exception of certain substrains of NZB that were considered Mta$^-$. Subsequently, reciprocal immunization procedures allowed

the development of cloned CTL lines recognizing an alternate form of Mta on NZB cells. The argument for mitochondrial control of Mta expression was substantially advanced by using these CTL. We have now demonstrated that the R6G-mediated loss of mitochondria from the BALB/c plasmacytoma fusion partner, x63/Ag8.653, and the concomitant loss of its Mta^a phenotype resulted in expression of the Mta^b phenotype of the NZB partner. Further, the mtDNA type of all treated hybrids that expressed the Mta^b type was that of the parental NZB cells. A causal relationship between mitochondria and Mta phenotype is further suggested by analysis of an exceptional R6G-treated hybrid that retained the BALB/c Mta^a type in that it was found to likewise have BALB/c-type mtDNA. Moreover, all of the control hybrids tested expressed only the Mta^a type of the BALB/c plasmacytoma parent, with the exception of one uncloned line that also displayed both mtDNA patterns. Coexpression of Mta^b of NZB origin was not detected in the control hybrids and restriction endonuclease analysis of mtDNA showed that only BALB/c-type mitochondria were present in Mta^a lines.

The finding of occasional hybrids that maintained the BALB/c Mta and mtDNA type despite R6G treatment was not unexpected because R6G may not have inactivated all mitochondria contributed by x63/Ag8.653. Yet, if a cytoplasmic element unrelated to mitochondria is also sensitive to R6G, it is improbable that such a factor would segregate together with both Mta and mtDNA type in every case. Indeed, if *Mtf* and mitochondria are not related, it is conceivable that the Mtf^{α} of R6G-treated hybrids could be inactivated and the cells thus express Mta^b in spite of the presence of BALB/c mitochondria. This was not observed in our sample. Indeed, in every hybrid tested, only one Mta determinant was expressed, and in every case the Mta phenotype correlated with the mtDNA phenotype of the cells.

Our finding that cloned hybrids did not express mtDNA or Mta of both types raises interesting, but as yet unresolved, questions. Although the mtDNA of many more lines would have to be analyzed to determine if cohabitation of BALB/c and NZB mitochondria can occur, there is precedent in the literature for mitochondrial incompatibility resulting in loss of one type (22–24). Indeed, one of the reported uses of R6G pretreatment of one partner is enhanced viability of the subsequent hybrids (14). We, and others (25), have reported previously that expression of Mta^a (Mta^+) is dominant in cell hybrids (9). However, the apparent dominance of Mta^a in our hybrids was due to the presence of only BALB/c-type mitochondria. Hybrids selected for maintenance of mitochondria of both types will be required to address the issue of phenotypic dominance.

Incompatibility of mitochondria themselves (14, 26), apart from the loss of chromosomes required for maintenance of one mitochondrial type, may suggest another cellular role for mitochondria as fundamental as the production of energy. Although the mitochondrial genome has been completely sequenced (20) and a number of gene products has been identified, several unidentified reading frames exist. If mitochondria have unidentified functional activities within the cell, affecting the antigenic structure of chromosomal gene products, it would be remarkable if Mta is the only molecule affected. It may be that this function is so highly conserved that only the most unusual crosses of animals within a species will produce changes that are antigenically distinct. Additionally, because most of the commonly used laboratory strains of mice have the same type of mitochondrial DNA, reflecting a common maternal ancestry (11), most cell surface antigens have not been tested in such a way that modulation by mitochondria would be detected. This problem is compounded because reciprocal F_1 female mice are generally regarded to be genetically identical, and because recognition of minor antigens such as Mta can only be detected when cytotoxic reactions to other more potent antigens are obviated (2, 27). Alternatively, it is possible that mitochondrial control of Mta is a vestigial function not relevant to other antigens. Whether the involvement of mitochondria in Mta expression is a vestigial role or one vital to cellular function, this interaction suggests previously unrecognized pathways of intracellular transport.

The molecular mechanism by which mitochondria control Mta phenotype also remains obscure. The possibility that the determinant was oligosaccharide-dependent through post-translational modification was investigated by treating targets of the C57BL/6 lymphoma line EL-4 ($Qa\text{-}1^b$, Mta^a) with tunicamycin to prevent N-linked glycosylation. A glycans-dependent $Qa\text{-}1^b$ determinant, detected by a CTL line with specificity for glycosylated $Qa\text{-}1^b$ molecules, was ablated by such treatment. In contrast, Mta^a expression was unaffected (30).

Previous studies on the cytoplasmic inheritance of Mta have utilized reciprocal F_1 hybrid and specific backcross mice. The use of mice to study Mta expression involves hypothetical concerns of maternal influence on gene expression during embryogenesis. Studies of the murine T^{hp} mutation suggest differential modification of this region of chromosome 17 in males and females with resulting developmental changes (28). Although maternal T^{hp} lethality has been shown to be a nuclear defect (28), its apparent maternal inheritance demonstrates how differential maternal and paternal gene activation during early development can influence and confound the identification of coding structures. The *in vitro* model system that we have employed for studying Mta expression circumvents hypothetical concerns of maternal effects during development. Importantly, the system described herein provides phenotypic markers for both the putative genome and the final antigenic type of a unique cell surface antigen, the structural gene for which is most likely encoded in the nucleus, but which is modified by, or recognized in conjunction with, a mitochondrial gene product.

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