Generation of T Cells with Lytic Specificity for Atypical Antigens. I. A Mitochondrial Antigen in the Rat

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Summary

F_1 rats primed with normal parental strain lymphocyte populations and restimulated in culture with parental lymphoblasts generate potent cytotoxic T cell responses to unusual antigen systems. Here we describe in the Lewis (L)/DA anti-DA combination an antigen system most likely of mitochondrial origin with the following properties: it is transmitted maternally from DA strain females, inherited in an extra-chromosomal manner, restricted by class I *RT1A*^{a} major histocompatibility complex gene products, extinguished on target cells treated with chloramphenicol, and its pattern of expression in different rat strains correlates with restriction fragment–length polymorphisms of mitochondrial DNA. Sequence analysis of the rat *ND1* gene indicates that the maternally transferred factor in the rat is not a homologue of the maternally transmitted factor responsible for the mitochondrial antigen in mice. In keeping with its inheritance from DA females, this antigen is present on target cells from (DA female × L male)F_1 donors and all other F_1 combinations derived from DA female parents, but absent from target cells from some F_1 combinations (L/DA and Wistar-Furth [WF]/DA) derived from DA strain males. The presence of this antigen in other F_1 combinations (Brown Norway [BN]/DA, August 2880 [AUG]/DA, and PVG/DA) indicates that this mitochondrial antigen system is shared by the DA, BN, and PVG strains, but not by the L and WF strains.

As a general rule, F_1 animals derived from matings between inbred strains do not react to cells or tissue grafts of parental origin. Usually, parental strain alloantigens are expressed codominantly in F_1 offspring; thus, for reasons of self tolerance, these antigens fail to provoke responses in F_1 hosts. Interesting exceptions to this rule are responses to the sex-linked antigen (H-Y) of mice and rats (1, 2), the hematopoietic histocompatibility antigen (Hh)[1] of mice (3, 4), the maternally transmitted antigen (Mta) of mice (5), idiotypic markers of parental TCRs in both species (6, 7), as well as certain other antigen systems not yet well defined (8, 9).

Analysis of some of these examples of F_1 antiparental reactivity indicates that CTL are involved in the response (10) and that the target antigen is clearly lacking in the F_1 responder. For example, expression of Mta depends on *Hmt*, an MHC-linked class I–like gene (5, 11, 12), β_2 microglobulin (11), and maternally transmitted factor (Mtf), a mitochondrially-encoded peptide (13). Because mitochondrial DNA is maternally inherited (14), certain F_1 strain combinations lacking this peptide are able to respond to its presence in parental cells. It should be noted that CTL recognition of the murine Mta system differs in one important respect from that of more conventional antigen systems; the lytic response to the Mtf peptide is restricted by an MHC class I–like gene product rather than the prototypic class I H-2K, D, and L MHC molecules (10, 15).

During the course of experiments designed to produce cytotoxic T cells in F_1 rats against idiotypic determinants of alloreactive parental strain T cells, we noted in some strain combinations that the responding F_1 T cells showed lytic specificity for target lymphoblasts of parental origin and also for target cells from syngeneic F_1 donors derived from reciprocal matings. At least two different antigen systems appear to be involved. One (H), the subject of the next paper in this series (see accompanying paper II [16]), is present on target

[1] *Abbreviations used in this paper:* AUG, August 2880; BN, Brown Norway; CAP, chloramphenicol; Hh, hematopoietic histocompatibility; L, Lewis; WF, Wistar-Furth.

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cells that are homozygous for $RT1C^{av1}$ expression, a gene of the class I–like MHC region of the rat. The other (MTA), the subject of this report, is most likely of mitochondrial origin; it is transmitted maternally, inherited in an extrachromosomal manner, restricted by class I *RT1A* MHC gene products, sensitive to treatment with chloramphenicol, and its pattern of expression in different rat strains correlates with RFLP and sequence analyses of mitochondrial (mt) DNA. Conditions for generating highly potent CTL specific for these atypical antigen systems require (*a*) in vivo priming of F_1 recipients with antigen-bearing parental T cells that include subpopulations reactive to host alloantigens, a circumstance that appears to bypass otherwise ineffective helper responses to these antigens; and (*b*) in vitro restimulation with antigen-bearing lymphoblasts (see accompanying paper III [17]).

Materials and Methods

Animals. Throughout this paper, the female parent of an interstrain mating is designated first. The rat strains used in this study, along with their MHC haplotypes are indicated in Table 1. Throughout the text, we refer to rat strains in terms of alleles they express in the various MHC regions. Thus, DA ($RT1^{av1}$) = A^aB/D^aC^{av1}), or for brevity, *aaav1*. Rats of the DA strain were purchased from Bantin and Kingman (Freemont, CA); Lewis (L), Wistar-Furth (WF), and Brown Norway (BN) rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN); the PVG congenic and recombinant strains were derived from the animal colony maintained at the Institute for Animal Physiology and Genetics Research (Cambridge, UK); and rats of the August 2880 (AUG) strain as well as all interstrain F_1 animals were bred and maintained at our animal facilities in La Jolla.

Generation of Killer Cells. To generate killer cells from F_1 responders, lymph node cells (1.5×10^7 in 0.1 ml) from DA rats were injected into the hind foot pads of L/DA recipients; donors and recipients were of identical sex. Cell suspensions were prepared 10 d later from the enlarged popliteal lymph nodes of the recipients and stimulated in culture with irradiated (3,000 rad) parental lymph node cells previously activated for 3 d with Con A (3 μg/ml). Cultures consisted of 10^7 responder cells and 10^6 stimulator cells in upright T25 flasks (Corning Glass Works, Corning, NY) containing 10 ml of T cell medium (RPMI 1640 with FCS [8%; J.R. Scientific, Woodland, CA], Hepes [10 mM; Sigma Chemical Co., St. Louis, MO], 2-ME [50 μM; Bio-Rad Laboratories, Richmond, CA], Indomethacin [1 μM; Sigma Chemical Co.], and penicillin-streptomycin [5 U/ml and 50 μg/ml, respectively; M.A. Bioproducts, Walkersville, MD]) supplemented with α-methyl mannoside (50 mM; Sigma Chemical Co.). 5 d later, cells from these cultures were used either in lytic assays or restimulated to provide killer cells for repeat experiments. For restimulation, responder (2×10^6) and stimulator (2×10^6) cells were cultured for 4 d as described above. In all cases, stimulator cells were mitogen-activated blast cells; normal lymph node cell populations proved to be totally ineffective (see accompanying paper III [17]).

MHC-specific killer cells were generated by culturing lymph node responder cells (2.5×10^7) for 6 d with irradiated (3,000 rad) spleen stimulator cells (2.5×10^7) in upright T75 flasks containing 25 ml T cell medium supplemented with α-methyl mannoside (50 mM) and supernatant (10%) from Con A–stimulated splenic cell cultures. To remove erythrocytes, cells from one spleen were pelleted and resuspended for 5 min at room temperature in a 5-ml mixture of TRIS base (20 mM; Sigma Chemical Co.) and ammonium chloride (0.15 M) in distilled water adjusted to pH 7.2. The cells were then diluted in 15 ml T cell medium and washed three times before culture.

Table 1. *Rat Strains Used in This Study and Their RT1 MHC Haplotypes*

	RT1 region				
Strain	*A*		*B/D*		*C*
DA	*a*		*a*		*av1*
L	*l*		*l*		*l*
WF	*u*		*u*		*u*
BN	*n*		*n*		*n*
AUG	*c*		*c*		*c*
PVG	*c*		*c*		*c*
PVG-$RT1^{av1}$	*a*		*a*		*av1*
PVG-$RT1^{u}$	*u*		*u*		*u*
PVG.R1	*a*	x	*c*		*c*
PVG.R8	*a*	x	*u*		*u*
PVG.R19	*a*		*a*	x	*c*
PVG.R20	*c*		*c*	x	*av1*
PVG.R23	*u*	x	*a*		*av1*

Frozen Cell Storage. In some instances, Con A–activated cells were frozen, stored at −70°C, and used as target cells after thawing. For freezing, 1 ml of freezing medium (FCS containing 10% DMSO, 3% glucose, and 25% RPMI 1640) was added to pellets of $1–10 \times 10^6$ cells; the medium was added ice cold and the cells maintained at 4°C for 60 min before storage in a styrofoam container at −70°C. Cells were thawed slowly by adding 10 vol of ice-cold T cell medium; they were then cultured overnight in media supplemented with CASUP (10%) and α-methyl mannoside (50 mM).

Lytic Assays. Target cells ($1–3 \times 10^6$) were pelleted, then resuspended in one drop of FCS and 100 μCi ^{51}Cr (0.1 ml), and incubated at 37°C with gentle agitation for 1–2 h. Generally, 5–10 $\times 10^3$ targets were used per well. Duplicate or triplicate threefold dilutions of killer cells were added starting at E/T ratios of 30:1 or 60:1. For cold target competition assays, effector/hot target cell ratios were 30:1, and dilutions of cold, unlabeled target cells were added in triplicate at cold/hot target cell ratios starting at 80:1. Percent specific lysis was calculated from the equation: 100× (experimental − spontaneous release)/(maximum uptake − spontaneous release). Generally, maximum uptake values were in the range of 800–3,000 cpm, and spontaneous release values were 10–20%.

Reagents. Chloramphenicol (CAP) was added to target cells in a concentration of 100 μg/ml for 24 h before labeling with ^{51}Cr. MAC 30 is a rat monoclonal alloantibody of the IgG2c isotype specific for $RT1A^a$; it has been used in the past to block lysis by killer cells specific for or restricted to this class I gene product (18). R3-40, a rat mAb standard of the same isotype (Pharmingen, San Diego, CA) was used as control. Both of these antibodies were used in fivefold dilutions starting at 12 μg/ml.

RFLP Analysis of mtDNA. Total cellular DNA was isolated from spleen and kidney. 6–8 μg DNA was digested with restric-

tion enzymes, subjected to electrophoresis on 0.7% or 1% agarose gels, and transferred to nylon membranes (19). Blots were hybridized with ^{32}P-labeled (20) mouse mtDNA (BamHI/PstI fragments contained in pUC 18 plasmids; Yonekawa, H., E. Hermel, and K. Fischer Lindahl, manuscript in preparation) and cohybridized with an excess of unlabeled pUC 18. Two post-hybridization washes were performed in 2× SSC for 5 min at room temperature, followed by two washes in 40 mM sodium phosphate (pH 7.2), 0.1% SDS for 20 min at 65°C.

Polymerase Chain Reaction (PCR). Amplification of the 5′ end of the *NDI* gene was conducted by PCR (21). Oligonucleotide primers corresponded to sequences at the 16S rRNA/tRNA^{LEU} junction (AA**ACTGCAG**CCCAAGTTATTAGGGTGG; nt 2646–2673, with the introduced PstI site highlighted) and within the *NDI* gene (CCC**GGATCC**AGAGGGTTGGGGCGAT; nt 2944–2960, with introduced BamHI site highlighted) (22). Amplifications were carried out in reaction volumes of 25 μl containing 50 pM of each oligonucleotide primer, reverse transcriptase buffer (23), 125 μM of each deoxynucleotide triphosphate, 5–10 ng rat total cellular DNA, and 1 U *Taq* polymerase (Cetus Corp., Emeryville, CA) under 50 μl mineral oil. The PCR reaction was carried out for 30 cycles with denaturation and extension steps of 94°C for 1 min and 65°C for 3 min, respectively.

Sequence Analysis of mtDNA. Reaction products were purified by ethanol precipitation and digested with BamHI and PstI. The digested DNA was subjected to electrophoresis in 2% Nusieve/1% GTG agarose (FMC), isolated from the gel, and cloned into pUC 18. Template DNA was amplified twice, and two positive clones from each amplification were sequenced to identify and avoid misincorporation artifacts. Double-stranded DNA was sequenced by the dideoxy chain termination technique (24, 25). Both the coding and noncoding DNA strands were sequenced.

Results

A Maternally Transmitted Antigen System Detected by CTL. L/DA anti-DA CTL were tested for their lytic activity on a panel of ^{51}Cr-labeled target cells from various strains and their F_1 hybrid combinations (Fig. 1, *A* and *B*). Two findings were unexpected: (*a*) targets from parental strain DA donors were lysed, but those from other homozygous donors, L, BN, AUG, and WF, were not; and (*b*) target cells from hybrid progeny of DA females or males were also lysed, except in two strain combinations, L/DA and WF/DA, where target cells from hybrid progeny of DA males were unaffected. As controls, MHC-specific CTL (e.g., DA anti-L) were used to demonstrate that target cells not lysed by L/DA anti-DA CTL were susceptible to lysis by CTL of appropriate specificity (data not shown).

Thus, DA/L and DA/WF animals, progeny of DA mothers, appear to express a maternally transmitted antigen system, which we have provisionally termed MTA, that is lacking in offspring of reciprocal F_1 matings to DA males. The presence of this antigen in other F_1 combinations, BN/DA and AUG/DA, suggests the possibility that the maternally transmitted component of this antigen in DA females may also be transmitted by BN and AUG females, but not by L and WF females. Evidence that the killer cells that detect this antigen system are T cells is provided by the finding that lysis is blocked by R73, a mAb specific for the rat TCR-α/β (26) (see accompanying paper III [17]).

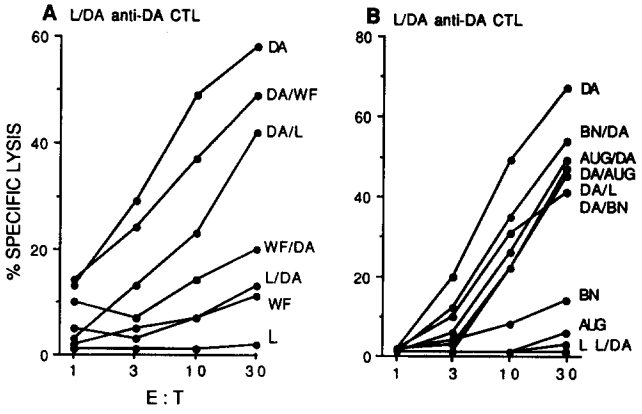

Figure 1. Lytic activity of L/DA anti-DA CTL against ^{51}Cr-labeled target cells of various origin. Except those from L/DA and WF/DA donors, all *RT1^{av1}*$^+$ target cells are lysed. Evidently, MTA expression on F_1 target cells depends upon a MTF inherited from DA strain females that is also present in AUG and BN females, and absent in L and WF females.

The results shown in Fig. 2, *A–C* confirm this maternal effect with cold target competition analysis; unlabeled L/DA, WF/DA, BN, AUG, and L lymphoblasts fail to block lysis of labeled DA/L target cells, while lymphoblasts from any other F_1 combinations involving DA matings blocked lysis. Direct lysis and cold target competition were assayed in >100 different experiments. On some occasions, WF and WF/DA target cells were lysed by L/DA anti-DA CTL well over background levels, but never to the extent that DA, DA/L, or DA/WF target cells were lysed (see example in Fig. 6). Also, while WF and WF/DA cells sometimes showed some lysis, they were never effective in cold target competition assays with DA/L target cells (data not shown).

Requirements for the RT1^{av1} Haplotype: Backcross Studies. Fig. 3 shows the results of three experiments designed to assess the role of the *RT1^{av1}* MHC haplotype in the maternally controlled expression in DA/WF targets of the MTA antigen detected by L/DA anti-DA CTL. A panel of 33 backcross progeny of DA/WF × WF (*av1/u* × *u/u*) matings were typed for expression of the segregating *RT1^{av1}* haplotype by: (*a*) MLC responses to *RT1^{av1}* stimulators; (*b*) target cell lysis by L anti-DA (*l* anti-*av1*) MHC-specific CTL; and (*c*) flow cytometric analysis with L anti-DA alloantisera or with MAC 30, a mAb specific for the class I *RT1A^a* gene product of DA rats (18). A total of 14 of these backcross donors were typed to be *RT1^{av1}*$^+$ by these three assays, and all of the 14 animals were MTA$^+$, their target cells being susceptible to lysis by L/DA anti-DA CTL; no individual was found to be *RT1^{av1}*$^+$, MTA$^-$, or *RT1^{av1}*$^-$, MTA$^+$. We conclude from this analysis that expression of MTA requires the *RT1^{av1}* haplotype. Such a lack of segregation of MTA and *RT1^{av1}* indicates that MTA is either: (*a*) the product of a gene(s) encoded within MHC or closely linked to it, whose expression is maternally controlled; or (*b*) that it is a maternally inherited, extra-chromosomal product, perhaps of mitochondrial origin, recognized by CTL in the context of *RT1^{av1}* MHC gene products.

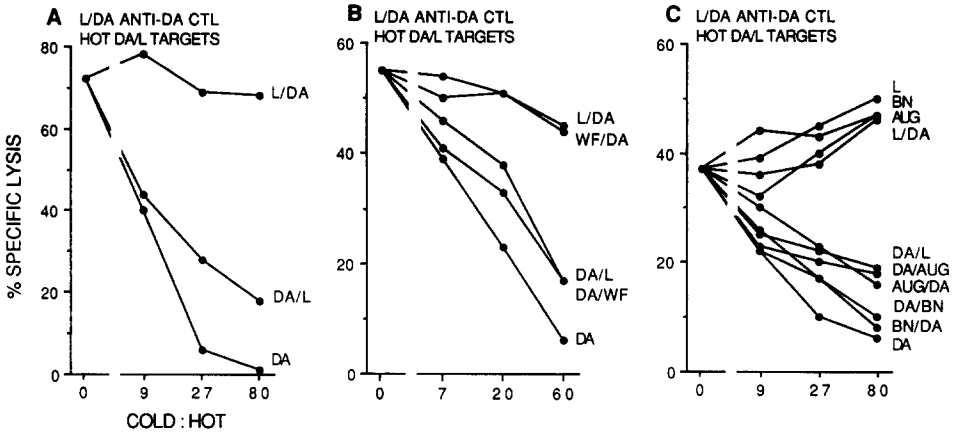

Figure 2. All target cells susceptible to lysis by L/DA anti-DA CTL effectively block lysis of labeled DA/L target cells in cold target competition assays.

MHC Restriction of MTA: Analysis of Intra-MHC Recombinants. Two elements seem to be required for expression of MTA: (*a*) a maternally transferred factor derived from females of the DA, BN, AUG, and PVG (see below) strains, but not from the L or WF strains; and (*b*) the presence of the $RT1^{av1}$ MHC haplotype. Previous efforts by one of our groups (G.W. Butcher, et al.) have generated a series of intra-MHC recombinants involving the $RT1^{av1}$, $RT1^{u}$, and $RT1^{c}$ haplotypes on the PVG background; these recombinants define the major class I (*RT1A*), class II (*RT1B/D*), and class I-like (*RT1C*) regions of the rat MHC locus (27). To identify the RT1 region required for MTA expression with these recombinant strains, it was first necessary to determine whether PVG-$RT1^{av1}$ rats express MTA. Several experiments indicated that this is the case; cold target cells from DA and DA/L donors, but not from L/DA donors, block lysis of labeled PVG-$RT1^{av1}$ target cells (data not shown).

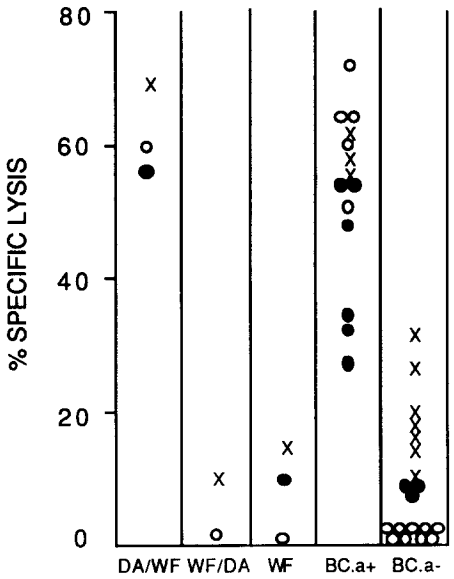

Figure 3. Expression of MTA requires the $RT1^{av1}$ haplotype. L/DA anti-DA CTL were incubated with ^{51}Cr-labeled target cells from DA/WF, WF/DA, WF, BC.a^+ and BC.a^- donors. BC.a^+ and BC.a^- animals are $RT1^{av1+}$ and $RT1^{av1-}$ backcross (BC) progeny of matings between DA/WF female and WF male rats. The three different symbols (X, ●, and O) represent three different experiments. 33 different backcross progeny were derived from four matings.

This finding permitted us the next step of assessing the expression of MTA on target cells of the PVG MHC congenic strains (*ccc*, *aaav1*, and *uuu*), as well as the intra-MHC recombinant PVG strains (*acc*, *aac*, *auu*, *ccav1*, and *uaav1*), both by direct lysis using L/DA anti-DA CTL (Fig. 4 *A*) and cold target competition assays with labeled DA/L target cells (Fig. 4 *B*). Thus, these experiments clearly demonstrate a requirement for the *a* allele of the *RT1A* region for expression of MTA; cells from donors expressing the *ccav1* or *uaav1* haplotypes on the PVG background were not lysed, nor were target cells from MTF negative WF/DA and L/DA donors, which express the *aaav1* haplotype.

Further Evidence for $RT1A^a$ Involvement in MTA Expression: Inhibition of Lysis with a mAb Specific for $RT1A^a$. MAC 30 is a rat mAb specific for the $RT1A^a$ molecules (18). Fig. 5 *A* shows that its presence markedly inhibits lysis of DA/L target cells by L/DA anti-DA CTL; as expected, it also inhibits MHC-specific lysis of DA/L target cells by L/WF (*lll/uuu*) anti-DA (*aaav1*) CTL (Fig. 5 *B*). This antibody fails to block the lytic effects of MHC-specific DA/L (*aaav1/lll*) CTL on WF (*uuu*) target cells (Fig. 5 *C*), suggesting that its inhibitory effects on anti-MTA lysis occur at the target cell level and not by inhibiting the killer cell directly.

Extra-chromosomal Inheritance of MTA. Studies described above demonstrate that lack of segregation of MTA and $RT1^{av1}$ in backcross offspring of DA/WF ($RT1^{av1/u}$) × WF ($RT1^{u}$) matings. The results presented in Fig. 6 indicate the basis for this lack of segregation; the maternally transmitted component of the MTA target structure detected by L/DA anti-DA CTL is inherited extra-chromosomally. Female $RT1^{u}$ ($RT1^{av1-}$, MTA$^-$) offspring of the backcross mating described above and in Fig. 3, derived ultimately from a DA female ancestor, were mated to WF/DA ($RT1^{u/av1}$, MTA$^-$) males. Of 13 progeny typed, six were $RT1^{av1+}$ and their target cells were lysed by L/DA anti-DA CTL; target cells from the remaining seven $RT1^{u/u}$ progeny were not. Therefore, a maternally transferred factor derived from a DA female ancestor, whose product is not recognizable in the context of the backcross $RT1^{u}$ haplotype, is again detectable in

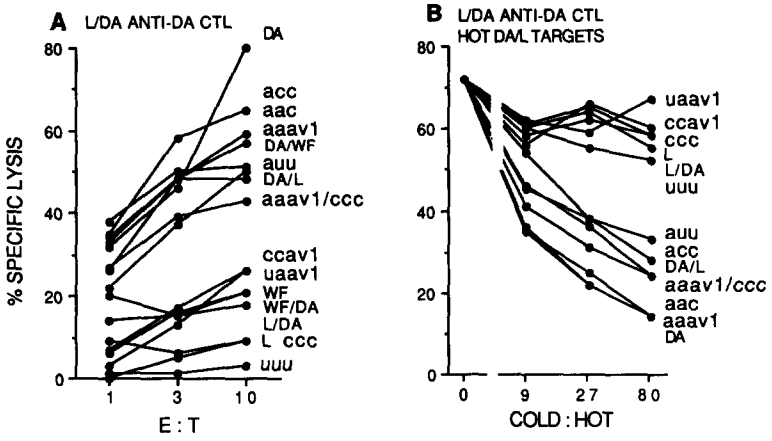

Figure 4. The presence of MTA on target cells requires expression of the *a* allele of the class I *RT1A* MHC locus. L/DA anti-DA CTL were (*A*) tested for lysis on ^{51}Cr-labeled target cells from various sources, including the MHC congenic and recombinant strains on a PVG background, or (*B*) tested in cold target competition assays on labeled DA/L target cells with unlabeled target cells from these various sources.

cells from progeny of a subsequent testcross mating where $RT1^{av1}$ is introduced from an MTA$^-$ WF/DA male parent.

Chloramphenicol Sensitivity of MTA. The precedent already established in mice of a maternally inherited antigen system determined by mtDNA (13), detectable with CTL (10), and sensitive to treatment with CAP (28) raises the question whether the present MTA system in rats might be similar. To explore this possibility, DA/L target cells were treated with CAP for 24 h before labeling with ^{51}Cr. CAP is a selective inhibitor of mitochondrial, but not of cytoplasmic, translation (29). CAP treatment of DA/L target cells inhibited lysis by L/DA anti-DA CTL (Fig. 7 *A*), but, as expected, CAP treatment did not inhibit lysis of DA/L target cells by DA anti-L CTL (Fig. 7 *B*). Thus, the results of this experiment support the extra-chromosomal inheritance of MTA and suggest that this antigen is either of mitochondrial origin or that its expression depends on mitochondrial protein synthesis.

Correlation of MTA Expression with mtDNA Haplotypes. In the mouse, Mta depends upon a mitochondrial gene locus (*Mtf*) (13), and alleles of *Mtf* correlate with RFLPs (30, 31). To determine whether this might be so for MTA of rats, we examined mtDNA from four MTF$^+$ rat strains (DA, BN, AUG, and PVG), two MTF$^-$ strains (L, WF), and an MTA$^+$ DA/WF × WF backcross donor as a control. Fig. 8 shows that three RFLP haplotypes could be detected after digestion of mtDNA with EcoRI, HhaI, and HincII. Digestions with BamHI, BclI, HindIII, MspI, and PstI failed to reveal any DNA polymorphisms.

The EcoRI digestions yielded the *A* and *B* patterns described by others (32, 33). The MTF$^+$ strains DA, AUG, and PVG displayed the *A* pattern, while the MTF$^-$ strains L and WF fell into the *B* family. The MTA$^+$ backcross control (DA/WF × WF) also expressed the *A* haplotype, as would be expected from maternal inheritance of mtDNA (14). A third mtDNA haplotype is revealed in the MTF$^+$ BN strain; it shares the EcoRI *A*, *B*, and *C* bands with DA and the combined *D*+*E* band with MTF$^-$ L and WF strains.

Comparison of the HhaI digests shows that the MTF$^+$ strains DA, AUG, PVG, and BN share one pattern, while MTF$^-$ L and WF mtDN revealed a second pattern. In contrast, HincII digests of BN mtDNA gave a pattern similar to that of L and WF. It therefore seems clear that the MTF$^-$ strains L and WF have a lineage of mtDNA distinct from the MTF$^+$ DA, AUG, and PVG strains, while BN mtDNA shares features of both.

Sequence of the Rat Mitochondrial ND1 Gene. Analysis of rat-mouse somatic cell hybrids demonstrated that rat mitochondria produce a factor that crossreacts with the mouse Mtf^a peptide (Loveland, B. E., J. Rolf, Y. Murata, H. Yone-

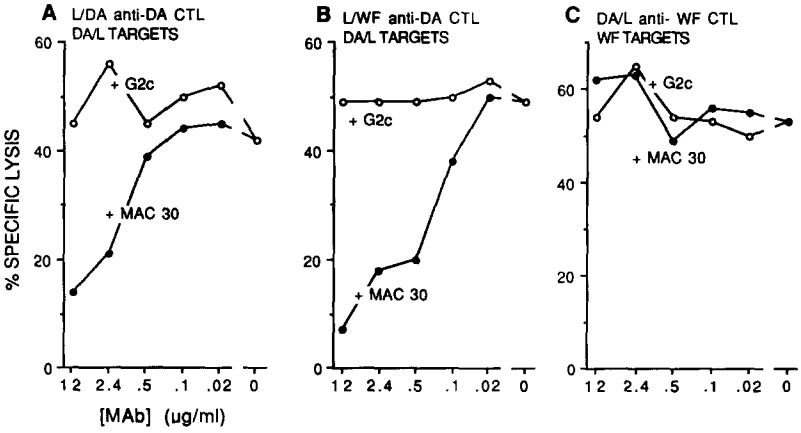

Figure 5. Further evidence of $RT1A^a$ involvement in MTA expression. (*A*) MAC 30, a mAB specific for $RT1A^a$ gene products, blocks lysis of DA/L target cells by anti-MTA CTL. For control purposes, this same antibody (*B*) blocks lysis of DA/L target cells (*aaav1/lll*) by class I-specific L/WF anti-DA CTL, but (*C*) does not inhibit lysis of WF target cells (*uuu*) by DA/L CTL. This latter finding indicates that the inhibitory effects of MAC 30 are not exerted at the CTL level. A control mAb of the same isotype, but different specificity (see Materials and Methods) has no effect.

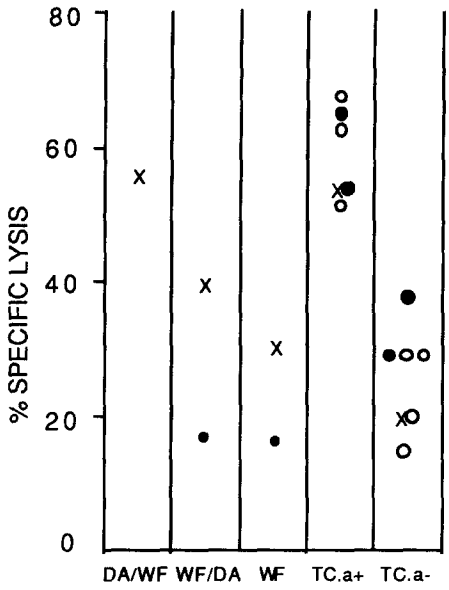

Figure 6. Expression of MTA involves a maternal component that is inherited extra-chromosomally. L/DA anti-DA CTL were tested for lysis on DA/WF, WF/DA, and WF target cells, and on target cells from testcross (TC) progeny of $RT1^{av1-}$ DA/WF × WF females (BC.a^- in Figs. 3) and WF/DA males. This testcross was designed to provide progeny from matings of MTF$^+$, MTA$^-$, $RT1^{av1-}$ females and MTF$^-$, MTA$^-$, $RT1^{av1+}$ males to determine whether an extra-chromosomally inherited component (MTF) in the female lineage, together with the paternally inherited $RT1^{av1}$ haplotype, would lead to the re-expression of MTA. The three different symbols (X, ●, and O) represent three different experiments. The testcross animals were derived from three different parental pairs.

kawa, and K. Fischer Lindahl, manuscript in preparation). This factor is most likely the NH_2 terminus of the rat ND1 protein, which is nearly identical to the mouse homologue for the first 38 amino acids, including an isoleucine at the critical position 6; the differences are Phe → Tyr in position 2 and Val → Ile in position 11 (mouse → rat) (34). To determine whether the mitochondrial ligand detected by L/DA anti-DA CTL is derived from the rat *ND1* gene, we sequenced the 5′ end of this mitochondrial gene from L, DA, and BN rats (Fig. 9). From nucleotide 2728 to 2961, including the first 78 codons of the *ND1* gene, we found two silent base changes, shared by L and BN but not by DA, but no amino acid differences between the three strains. Therefore, we conclude that the rat MTF is not a homologue of the mouse Mtf, and that it is encoded elsewhere in the mitochondrial genome.

Discussion

Previous studies by one of our groups have defined a mitochondrially inherited antigen system in mice, peptides of which are recognized by CTL restricted to medial or class I-like gene products of the H-2 complex (35). The particularly interesting feature of this murine antigen system is that gene products of the major class I loci (*K*, *D*, and *L*) are not involved in the presentation and recognition of this antigen. The present studies describe for the first time in rats an antigen system that is also extra-chromosomally inherited and maternally transmitted, but unlike the mouse, recognition of this antigen by CTL is restricted to classical class I MHC gene products of the *RT1A* region.

This antigen system was first found by "priming" L/DA F1 rats in vivo with parental strain DA lymphocytes under conditions of a local GVH reaction and then restimulating these F_1 lymphocytes in culture with irradiated lymphoblasts of the same parental strain. These primed and restimulated L/DA anti-DA cells were shown to lyse $RT1^{av1+}$ target cells from homozygous donors (DA and PVG.$RT1^{av1}$; Fig. 1, *A* and *B*, and 4 *A*) as well as $RT1^{av1+}$ target cells from F_1 progeny of some strain combinations (DA/BN, DA/AUG, BN/DA, and AUG/DA) regardless of whether the $RT1^{av1}$ haplotype was derived from the male or female parent. For other $RT1^{av1+}$ F_1 target cells (DA/L and DA/WF), however, lysis occurred only if DA females were used in the interstrain mating; target cells from $RT1^{av1-}$ strains (L, BN, AUG, WF, and PVG) and from certain $RT1^{av1+}$ F_1 donors, where DA was the male parent (L/DA, and WF/DA), were not lysed (Fig. 1, *A* and *B*, and 4 *A*). These results were confirmed in all instances by cold target competition assays; target cells not susceptible to lysis by L/DA anti-DA CTL failed to block lysis of labeled DA/L target cells (Fig. 2, *A–C*, and 4 *B*). The simplest interpretation of these findings is that this antigen system depends upon a maternally transferred factor shared in the DA, BN, AUG, and PVG strains, and is different or is absent in L and WF. It is interesting to note that there is evidence from grafting experiments for a maternally transmitted histocompatibility antigen difference between L and BN (V. Kren, personal communication). Expression of this antigen system depends upon the $RT1^{av1}$ haplotype (Fig. 3), in particular, the class I $RT1A^a$ gene product (Fig. 4, *A* and *B*); confirmation of this is provided by the finding that an anti-RT1A^a mAb effectively blocks lysis of target cells (Fig. 5 *A*).

Several lines of evidence support the conclusion that this

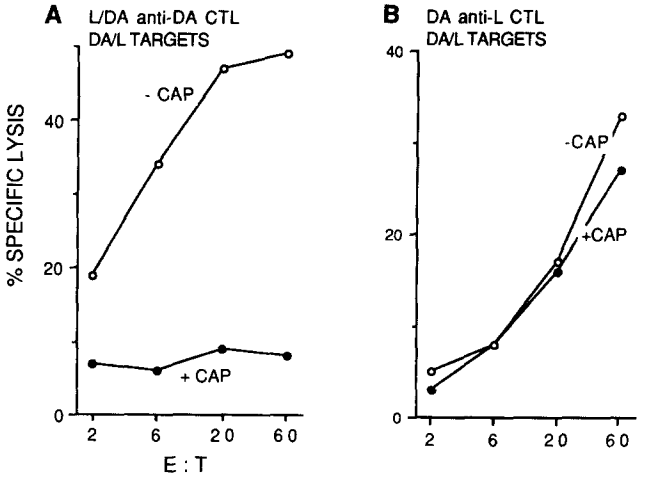

Figure 7. The expression of MTA depends upon mitochondrial translation. DA/L target cells were incubated with or without 100 μg/ml chloramphenicol (CAP) for 24 h before labeling with ^{51}Cr, and then exposed to (*A*) L/DA anti-DA CTL and (*B*) MHC-specific DA anti-L CTL.

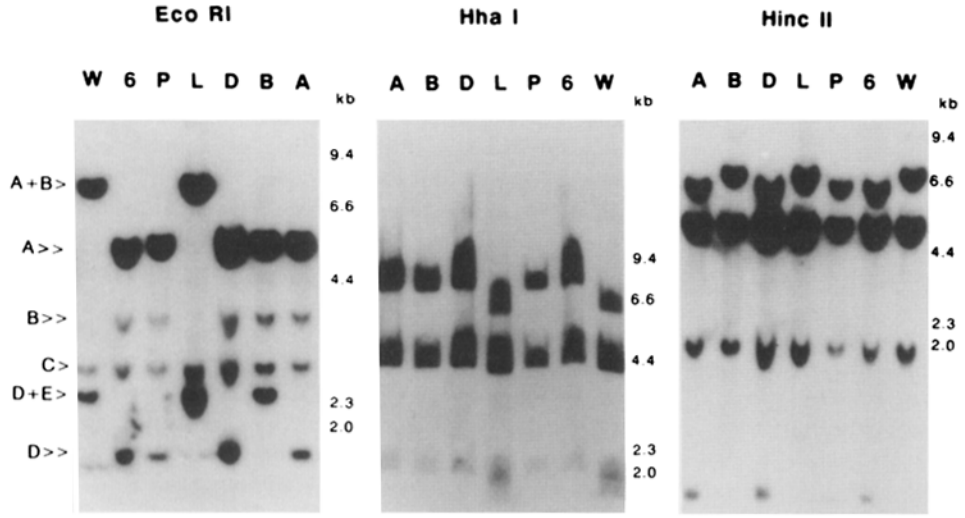

Figure 8. Southern blot analysis of rat total cellular DNA digested with the indicated endonucleases and hybridized with mouse mtDNA. Lane *A*, AUG; lane *B*, BN; lane *D*, DA; lane *L*, Lewis; lane *P*, PVG; lane *6*, DA/WF × WF backcross; lane *W*, WF. EcoRI bands are indicated at the far left. Band *E* has run off the gel.

antigen system is either of mitochondrial origin or that it depends on a particular allele of a polymorphic locus of mtDNA. It is extra-chromosomally inherited (Fig. 6); it is extinguished on target cells after treatment with chloramphenicol, an inhibitor of mtRNA translation (Fig. 7); and its expression correlates with RFLPs of mtDNA (Fig. 8). The murine Mtf is derived from the NH_2 terminus of the mitochondrial gene *ND1* (13). However, the rat MTF appears not to be a homologue of the murine Mtf; sequence lysis of the rat mitochondrial *ND1* gene revealed no predicted amino acid differences among the MTF^+ DA and BN rat strains and the MTF^- L strain (Fig. 9).

There are several ways to find the rat MTF. From the restriction maps of rat mtDNA (36, 37), it is possible to locate the polymorphic restriction sites that we detected in L, DA, and BN mtDNA. Both L and WF have lost an EcoRI site from the junction of the ribosomal RNA genes, and they

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                                                          ←tRNA^Leu
.....CAGAGCCAAGTAATTGCGTAAGACTTAAAACCTTGTTCCCAGAGGTTCAAATCCTCTCCCTAATA
          .         .         .    2700.         .         .

ND1→
M  Y  F  I  N  I  L  T  L  L  I  P  I  L  I  A  M  A  F  L  T  L  V  E  R
GTGTACTTTATTAATATCCTAACACTCCTAATCCCAATCTTAATTGCCAT*GGCCTTCCTCACCCTAGTAGAACGG
 .         .    2750.         .         .         .         .    2800.

K  I  L  G  Y  M  Q  L  R  K  G  P  N  N  E  G  P  Y  G  K  L  Q  P  F  A
AAAATCCTAGGCTACATACAATTACGCAAAGGCCCCAACAACGAAGGCCCATATGGTAAACTACAACCATTTGCA
          .         .    ↓    .         .    2850.         .         .
                         C

D  A  M  K  L  F  M  K  E  P  M  R  P  L  T  T  S  M  S  L  F  I
GATGCCATAAAACTATTCATAAAAGAACCCATACGCCCTCTAACCACCTCAATATCACTATTTATC...
 .         .    2900.         .    ↓    .         .         .
                                   C
```

Figure 9. Sequence of the PCR-amplified 5′ end of BN, DA, and L rat *ND1*. Nucleotide positions correspond with the published type A *Rattus norvegicus* sequence (22). The asterisk marks the missing A nucleotide at 2779, while the additional C nucleotide at 2785–2786 is underlined. The first GTG codon, which has been proposed to act as a start codon in rodents, is shown as encoding methionine (13, 58). Nucleic acid differences, causing silent changes in the BN and L strains, are indicated at positions 2825 and 2917. These sequence data are available from EMBL/GenBank/DDBJ under accession number X56833.

have gained an HhaI site in the *COI* or *Cyt b* gene, while L, WF, and BN have lost an EcoRI site in the *ND1* gene, as well as a HincII site in the noncoding D loop. In addition, we have found that the 5′ end of the *ND1* gene is shared by L and BN.

This information is not sufficient to identify an area of the mitochondrial genome likely to encode MTF. For this, one may search for this gene using methods similar to those used in the past to find the mouse *Mtf* gene (13); by sequencing ~11 kb of mtDNA that encodes protein from strains L and BN, and comparing this with the published sequence for type A mtDNA (typical of the DA strain) (22), positions where DA and BN are identical and differ from L can be identified. Peptides having the sequences surrounding each of these positions can then be synthesized and tested for their ability to mimic the DA MTF antigen when added to L/DA target cells.

An alternative approach used in the past by Rammensee and colleagues (38, 39) could also be considered; this would involve isolating mitochondrially encoded proteins of DA rats (first in the form of complex I, cytochrome b, cytochrome oxidase, and F1-ATPase), and assaying peptide digests for their ability to mimic MTF. Once a single active protein was identified, the candidate gene could be sequenced and synthetic peptides tested as above.

The mouse Mtf peptide has a unique association with the restriction element, Hmt. To date, we have found no evidence that Mtf can be presented by the major class I restriction elements of the mouse *H-2* locus; similarly Hmt has not been shown to present any other peptide antigen to T cells (35, 40). Because of this unique association, it has been suggested the Hmt class I–like molecule might have special properties not found with the prototypical class I restriction elements, properties that would allow it to interact preferentially with peptide ligands of mitochondrial origin, perhaps as part of an intracellular transport mechanism (41). However, the current studies with the rat MTA system showing that class I RT1A molecules can also present mitochondrial peptide ligands argue against this notion.

An alternative possibility for the association between class I-like gene products and mitochondrial peptides seems worth considering. Since the murine Mtf is an NH_2-terminal peptide of a mitochondrially synthesized protein, it carries an *N*-formyl-methionine (13). Unlike peptide presentation to T cells by prototypical MHC molecules, this formyl group on the NH_2-terminal residue is essential for Hmt binding, whereas the methionine can be exchanged for phenylalanine or valine (42). This raises the possibility that Hmt might have a special pocket for binding peptides having an NH_2-terminal formyl group, thus making this restriction element specific for peptides of prokaryotic or mitochondrial origin (43). It remains to be determined whether the rat mitochondrial peptide MTF is also a formylated NH_2-terminal peptide.

The finding here that a rat mitochondrial peptide can be presented by a major class I MHC molecule supports the conclusion that any peptide may act as a minor histocompatibility antigen, irrespective of the cellular location or function of the protein from which it is derived (13). If immune surveillance has an important function in eliminating cells presenting mutant forms of endogenous proteins (44), the surface presentation of mitochondrial peptides would be functionally useful. The germline mutation state of mtDNA is ~10-fold higher than that of nuclear DNA (45), and mtDNA is a prime target for numerous chemical mutagens, such as ethidium bromide (46). However, it is questionable whether an immune surveillance mechanism could detect germline mtDNA mutations; the predominance of silent mutations is more likely explained by strong functional selection of proteins (47). Warburg (48), for example, proposed that neoplastic cells have an impaired respiratory capacity; while his prediction of a high glycolytic rate in cancer cells has been amply confirmed (49), a causal relationship between mitochondrial mutations and transformation has never been established (50).

It might be considered that immune surveillance can deal with mutations in somatic cells resulting from inaccurate replication, the influence of nuclear genes (51), or from the impact of environmental mutagens. Although this reasoning predicts a higher frequency of mutant mtDNA in tissue culture cells or immunodeficient individuals, the rate may nevertheless be insufficient for easy detection. Recently, it has been appreciated that a number of neuromuscular diseases are the result of deletions, duplications, and point mutations in the mitochondrial genome (52–54); some of these result in the synthesis of altered proteins, and the immune system could play a role in policing a selfish mitochondrial genome (55–57) or impose an autoimmune component to such diseases.

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