A *trans*-ACTING MAJOR HISTOCOMPATIBILITY COMPLEX-LINKED GENE WHOSE ALLELES DETERMINE GAIN AND LOSS CHANGES IN THE ANTIGENIC STRUCTURE OF A CLASSICAL CLASS I MOLECULE

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The classical class I major transplantation antigens play an essential role in the recognition of antigen by CTL. Their extreme polymorphism is presumably central to their function in binding peptide derivatives of rapidly evolving intracellular pathogens. All the evidence to date has indicated that this functional polymorphism depends solely on primary sequence polymorphism in class I H chain genes. First, such genes, and their protein products, are extensively polymorphic, especially in the $\alpha 1$ and $\alpha 2$ domains (1). Second, classical genetic experiments demonstrate that functional polymorphism of class I molecules segregates accurately with class I loci (2–5). Third, it has been shown repeatedly that the normal polymorphic structure of functional class I molecules can be reconstituted in cells by transfection of class I H chain genes alone (6, 7). Finally, functional polymorphism defined by the activity of alloreactive or MHC-restricted T cells has been referred explicitly to particular amino acid residues coded in the $\alpha 1$ and $\alpha 2$ domains of the H chain that appear to encircle the putative peptide-binding site (8).

In this study we report an exception to these general observations. We find that the alloantigenic structure of the product of the classical class I locus of the rat can be changed significantly by the action of a gene located elsewhere within the rat MHC. These structural changes are especially conspicuous at the level of primary alloreactive and secondary MHC-restricted cytotoxic T cells, but can also be detected with a monoclonal alloantibody.

This work was supported in part by U. S. Public Health Service grants AI-13162 and CA-34913 and by Medical Research Council (UK) grants g7904563C and g8121898CA. A. Livingstone was the recipient of a Research Studentship from the Medical Research Council (UK) during a part of this study, and S. Powis is the recipient of a Research Studentship from the Agriculture and Food Research Council. A. Livingstone's current address is the Division of Immunology, Department of Medicine, Stanford University Medical Center, Stanford, CA 94305, and A. G. Diamond's present address is the Department of Medicine, University of Newcastle upon Tyne Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK.

[1] *Abbreviations used in this paper:* CAS, Con A supernatant; CML, cell-mediated lympholysis assay.

The rat MHC, RT1, has been divided by recombination into several functionally distinct regions (Fig. 1) (9, 10). The RT1.A region codes for the H chain of classical class I molecules equivalent to mouse H-2K, -D, and -L. In terms of location in the MHC, RT1.A seems to be most closely related to mouse H-2K. RT1.A class I molecules are strong transplantation antigens, restrict cytotoxic T cell responses against viruses and minor histocompatibility antigens, induce potent alloantibody responses, and stimulate strong primary cytotoxic T cell responses in vitro. The adjacent class II region has been split into three subregions: RT1.B and RT1.D coding for molecules homologous to H-2 I-A and I-E, respectively (11, 12), and RT1.H containing DNA sequences that crosshybridize with human DP probes (13). The RT1.E and RT1.C regions contain the majority of rat class I genes, and specify a number of class I alloantigens with properties generally reminiscent of medial histocompatibility antigens of the Qa and Tla regions of the mouse MHC (14–18).

The present experiments show that the RT1.A classical class I molecules of the $RT1^a$ haplotype are subject to structural modification by the *trans*-acting product of a new locus, *cim*, mapping to the right of the RT1.A/RT1.B recombination site. Strains carrying $RT1.A^a$ and the dominant *cim* allele express $RT1.A^a$ class I molecules that are antigenically distinct from those expressed in strains where the dominant *cim* allele has been lost by recombination. That the modifying locus acts in *trans* can be demonstrated by reconstitution of the dominant antigenic phenotype in appropriate F_1 hybrids. Biochemical analysis shows that the modifier has a marked influence on the post-translational processing of $RT1.A^a$. The results are discussed in terms of the possible mode of action of this novel MHC-linked locus.

Materials and Methods

Animals. Rats were bred in the conventional animal colony in the Immunology Department, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, UK, or were obtained from OLAC 76 Ltd., Shaw's Farm, Bicester, Oxon, UK. The MHC haplotypes of the strains used in this study are given in Table I, where we also indicate the abbreviated strain names used in the text.

Immunizations. Female rats were immunized against the rat male antigen H-Y by a single injection of syngeneic male lymphoid cells. Spleens from male rats were prepared as a cell suspension in PBS, washed once, and resuspended in 10 ml PBS. Each female rat received 1 ml of this cell suspension: 0.1 ml injected subcutaneously at five sites (twice at the shoulders, twice at the flanks, and once at the base of tail), and 0.5 ml into the peritoneal cavity. Lymph node cells from primed rats were boosted with male cells in vitro at least 3 wk after immunization before being assayed for cytolytic activity.

Media. Cells were prepared and washed in RPMI-Hepes (RPMI 1640, Flow Laboratories, Irvine, UK, buffered with 2.5 mM Hepes (Sigma Chemical Co., St. Louis, MO), supplemented with 5% FCS (Sera-Lab, Crawley Down, Sussex, UK) for cells to be set up in culture, or with 10% Haemaccel (Veterinary grade; Hoechst UK Ltd., Hounslow, Middlesex, UK) for washing effector and target cells in cell-mediated cytotoxicity assays. Cells were cultured in complete medium: RPMI 1640 plus 5% FCS, 2.5×10^{-5} M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Con A Supernatant (CAS)[1]. Pooled spleen and lymph node cells from female PVG rats were cultured at 2.5×10^6 cells/ml in complete medium containing 2.5 μg/ml Con A (Sigma Chemical Co.). After 40–44 h, the supernatant was decanted, centrifuged at 800 *g* for 10 min, then at 2,000 *g* for 30 min, sterilized by passage through an 0.22-μ filter (Millipore Continental Water Systems, Bedford, MA), and stored at −20°C.

mAbs. Three mAbs specific for the $RT1.A^a$ class I molecule were used in this study.

R2/15S, R3/13, and JY3/84 are rat alloantibodies obtained from AO anti DA ($RT1^u$ anti-$RT1^a$) immunizations. They bind at three distinct sites, designated S, P, and T, respectively, on the $RT1.A^a$ molecule (20). R2/15S and JY3/84 are both IgG2a antibodies, while R3/13 is an IgG2b antibody. The R2/15S preparation used to block T cell-mediated cytotoxicity was purified by ammonium sulphate precipitation from 20-fold-concentrated serum-free culture supernatant. It was dialyzed extensively against PBS and then against RPMI-Hepes before use. Tissue culture supernatants of R2/15S and JY3/84 were used as first-stage antibodies for RIA.

Cytotoxic T Cells. Cytotoxic responses against MHC alloantigens were generated in primary MLC. Unprimed responder cells (lymph node or thoracic duct lymphocytes) were cultured with 3,300-rad gamma-irradiated (^{137}Cs source, Gammacell 40; Atomic Energy of Canada, Ottawa) stimulator cells (lymph node cells, or a mixture of lymph node plus spleen cells) in 200 μl medium in round-bottomed microtiter plates. Cells were incubated for 5 d, harvested, and assayed for cytotoxic activity. CTL specific for the rat male antigen H-Y were generated by the restimulation of lymph node cells from primed female rats. 10^5 responder lymphocytes were cultured with 3×10^5 irradiated (3,000 rad) male lymph node cells in 200 μl in round-bottomed microtiter wells as described above, harvested on day 5, and assayed. In many experiments, 25% CAS plus 50 mM α-methyl mannoside to prevent nonspecific T cell activation was added to the culture medium.

Targets. Lymph node cells from normal rats were cultured at 2.5×10^6 cells/ml in medium plus 2.5 μg/ml Con A. Blasts were harvested after 24–28 h, centrifuged at 200 *g* for 3 min, and the supernatant was discarded. Sodium ^{51}Cr-chromate in aqueous solution (CJS4, The Radiochemical Centre, Amersham, Bucks, UK), equivalent to 50 μCi, was added to the pelleted cells, which were then flicked into suspension. Targets were labeled for 1–4 h at room temperature, washed twice in RPMI-Hepes + 10% Haemaccel, resuspended in RPMI-Hepes + FCS, and counted.

Cell-mediated Lympholysis Assay (CML). Effector cells were harvested from MLC on day 5, pelleted by centrifugation at 200 *g* for 5 min, resuspended in RPMI-Hepes + 10% FCS, and counted by trypan blue dye exclusion. They were then made up to an appropriate concentration and serial threefold dilutions were made. 100 μl effector cells plus 100 μl ^{51}Cr-labeled target cells (10^5 cells/ml) were dispensed into round-bottomed microtiter wells; control wells for determining spontaneous release values received 100 μl targets plus 100 μl medium. Effector and target cells were mixed thoroughly and centrifuged (65 *g* for 2 min). The plates were incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2 in air. 100 μl supernatant was harvested from each well and counted in a gamma scintillation counter. Lysis was calculated according to the formula: percent cytotoxicity = 100 × (experimental counts − spontaneous release)/(total input counts − spontaneous release). Input counts were determined with appropriate aliquots of the target cell suspension. Spontaneous release values were generally 10–20% of total input counts. SDs of replicate wells are shown when >3% of the mean.

Cold Target Competition. Unlabeled (cold) Con A blasts were made up to 2×10^7 cells/ml in RPMI/Hepes + 10% FCS. Two further threefold dilutions were usually made, to give final cold/hot target ratios of 100:1, 33:1, and 11:1 in the assay. 50 μl cold targets, 50 μl ^{51}Cr-labeled targets (at 2×10^5 cells/ml), and 100 μl effector cells (or 100 μl medium in spontaneous release wells) were dispensed in that order into microtiter wells, mixed, centrifuged, incubated, and harvested as described above.

Inhibition of Lysis by mAbs. Serial fivefold dilutions of antibody from an initial concentration of 100 μg/ml were made in RPMI-Hepes + 10% FCS. 50 μl antibody plus 50 μl ^{51}Cr-labeled target cells (2×10^5 cells/ml) were dispensed into microtiter wells and incubated at room temperature for 30 min. 100 μl effector cells (or 100 μl medium in spontaneous release wells) were then added, effector and target cells mixed, and the assay was incubated for a further 4 h at 37°C before harvesting.

In many of the experiments in this study, the $RT1.A^a$ class I molecule was expressed on both effector and target cell populations. It has been shown that antibody specific for class I molecules expressed on both effector and target cells does not inhibit lysis (19). We confirmed this by assaying (a × c)F_1 anti-AO CTL (specific for $RT1^u$ alloantigens) on AO ($RT1^u$) and

(AO × DA)F_1 ($RT1^u$ × $RT1^a$) targets in the presence of the antibody R2/15S. In neither case was there any significant inhibition of lysis (data not shown).

RIA. The binding of mAbs R2/15S and JY3/84 to erythrocytes from various rat strains and F_1 hybrids was determined in a two-stage RIA using ^{125}I-rabbit anti-rat IgG(Fc) antiserum at the second stage, as described elsewhere (20).

Two-dimensional Peptic Peptide Maps. Peptide mapping was performed on class I H chains derived from Con A blasts surface iodinated using the lactoperoxidase method. The location of the appropriate bands was determined by autoradiography of the frozen SDS polyacrylamide gels, after which the gel was sectioned, and the labeled material eluted. Subsequent digestion with pepsin and separation of the peptides on silica gel thin layer elecrophoresis followed by chromatography was as previously described (21, 22).

Pulse-chase Labeling and Immunoprecipitation. Con A blasts were labeled for 10 min with L-[^{35}S] methionine (Amersham International, Amersham, UK) after a 30-min preincubation in methionine-free MEM (Gibco Laboratories, Paisley, UK). Incorporation was terminated by washing and subsequent reincubation of the cells in DMEM containing a fivefold excess of unlabeled methionine. Aliquots of cells were removed at the indicated post-pulse time points and the cells immediately pelleted and lysed in 200 μl lysis buffer (2% NP-40, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM PMSF). The lysates were kept on ice for 30 min, spun at 11,600 *g* for 6 min to remove debris, and stored at −20°C until immunoprecipitation. Cell lysates were precleared for 1 h at 4°C with 20 μl (packed volume) of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) coupled to an irrelevant rat mAb. RT1.A^a was then precipitated with 20 μl of R3/13 coupled to Sepharose 4B for 2 h at 4°C. The Sepharose reagent was washed twice in buffer (0.5% NP-40, 0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl, 10 mM EDTA) and boiled in 20 μl of SDS sample buffer (0.6 M Tris-HCl, pH 6.8, 2.3% SDS, 5% 2-ME, 10% glycerol) for 2 min. Supernatants were analyzed by SDS-PAGE on 11% gels. Gels were treated with Amplify (Amersham International), dried, and fluorographed at −70°C.

Results

CTL Specific for RT1.A^a Detect a Reciprocal Antigenic Difference between Parental and Recombinant Targets. The A^a class I molecule was the principal target for alloreactive CTL populations raised against a full *a* haplotype incompatibility or against an A^a difference alone (see Fig. 1 for MHC map and Table I for MHC haplotypes of strains used in this study and for abbreviated strain names underscored in the text). c anti-a CTL (a full *a* haplotype incompatibility) killed R1 and R8 targets almost as effectively as parental a targets, (Fig. 2 *a*), while c anti-R1 CTL (an A^a difference alone) killed all three target populations equally well (Fig. 2 *b*). The mAb R2/15S, specific for the A^a class I molecule, blocked the lysis of a, R1, and R8 targets by both CTL populations (Fig. 2, *c* and *d*), confirming this molecule as the principal target antigen for these cytotoxic responses. While R2/15S completely blocked the

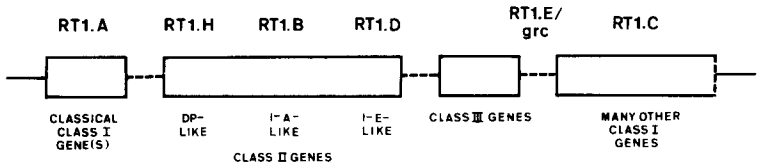

FIGURE 1. A diagram representing the regions of the rat MHC, RT1, on chromosome 14 (23). Most of the assignments are justified in published reviews (9, 10). The existence of a new class II sub-region to the left of RT1.B has been demonstrated recently (13). The proposed mapping of class III genes is implied by a recent study on the localization of C4 (24).

TABLE I

Rat Strains Used in this Study and their MHC Haplotypes

Strain	Abbreviated strain name*	RT1 haplotype	Origin of RT1 region A	B/D	C	MHC
Independent Inbreds						
AO	-	*u*	u	u	u	-
DA	-	*a*	a	a	a	-
PVG	c	*c*‡	c	c	c	-
MHC congenics						
PVG-RT1^{av1}(DA)	a	*a*	a	a	a	DA
PVG-RT1^{lv2}(AGUS)	l(AGUS)§	*l*	l	l	l	AGUS
PVG-RT1^{lv1}(F344)	l(F344)§	*l*	l	l	l	F344
PVG-RT1^{u}(AO)	u	*u*	u	u	u	AO
MHC recombinant congenics						
PVG.R1	R1	*r1*	a	c	c	DA/PVG
PVG.R7‖	R7	*r7*	a	c	c	DA/PVG
PVG.R8	R8	*r8*	a	u	u	DA/AO

* Abbreviated strain names have been used in the present text in the interests of clarity. Abbreviated strain names are underscored.

‡ To be precise, the DA strain carries the variant *av1* haplotype and has differences from the canonical *a* of the strain AVN, notably in the nonclassical class I genes of the C region. In the interest of clarity, however, we have used the name *a* for the MHC haplotype of DA. No AVN-derived rat strains were used in this study so no ambiguities arise.

§ Congenic strains bearing two independent variant *l* haplotypes *lv?*(AGUS) and *lv1*(F344).

‖ Strain now extinct.

lysis of all appropriate targets by c anti-R1 CTL (Fig. 2 *d*), and of R1 and R8 targets by c anti-a CTL (Fig. 2 *c*), it did not completely block the lysis of parental a targets by c anti-a CTL (Fig. 2 *c*), probably because a small component of this response was directed against antigens coded by the B^a, D^a, or C^a regions (25).

Unexpectedly, when c anti-a CTL were assayed on ^{51}Cr-labeled parental a targets in the presence of *a* haplotype (DA and a) or recombinant (R1, R7 and R8) cold targets, DA and parental a cold targets blocked lysis about ninefold more effectively than recombinant cold targets (Fig. 3 *a*). Cold target competition of lysis by c anti-R1 CTL gave the opposite pattern of inhibition. R1, R7, and R8 cold targets were about nine times more effective than either DA or parental a cold targets at blocking the lysis of ^{51}Cr-labeled targets by c anti-R1 CTL (Fig. 3 *b*). The reciprocal character of these results suggested that there was a gain and loss antigenic difference between the A^a class I molecules expressed on parental a cells and those expressed on cells from recombinant strains carrying only the A^a region of the *a* haplotype.

We next asked whether any such difference could be demonstrated with MHC-restricted CTL. RT1.A^a class I molecules have been shown to restrict cytotoxic responses to the male antigen H-Y (18). Anti-H-Y CTL were therefore generated in parental a and recombinant strains, and assayed on a and recombinant targets. Again, we observed a distinction between parental a and recombinant targets, which in this case was immediately apparent by direct cytotoxic assay. a anti-H-Y-specific CTL killed a strain male targets better than R1 or R8 male targets, while R1 anti-H-Y CTL killed R1 and R8 male targets better than a strain male targets (Fig. 4, *a* and *b*). Both a anti-H-Y CTL and R1 anti-H-Y CTL were restricted by the A^a class I mol-

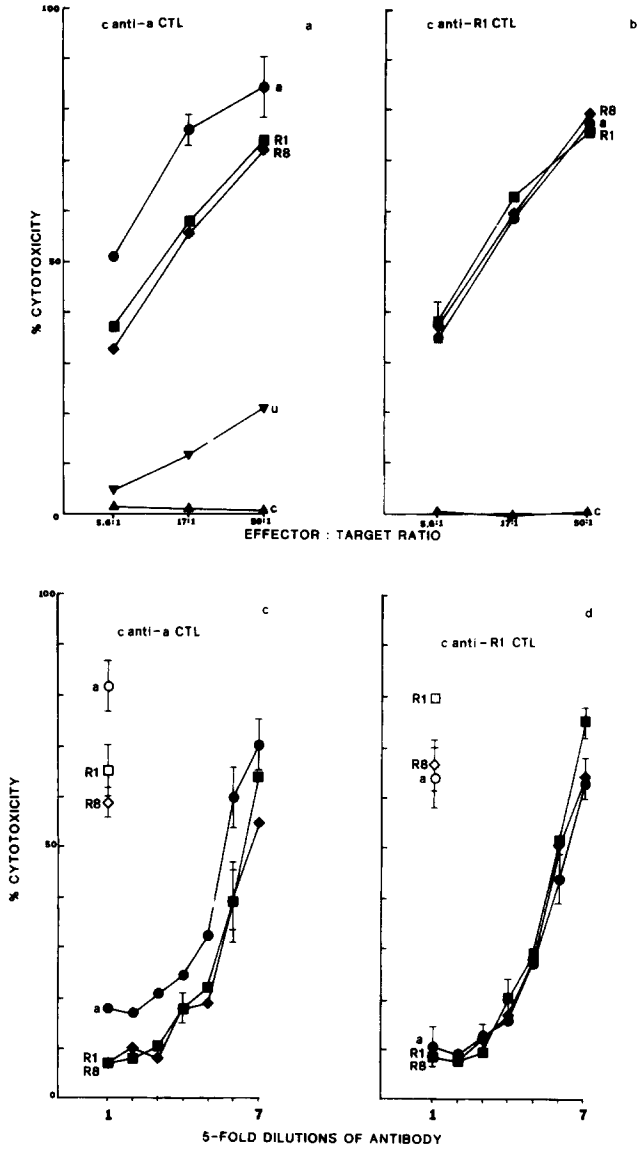

FIGURE 2. Alloreactive CTL raised against a full RT1^a incompatibility, or against an RT1.A^a difference alone, are largely specific for the A^a class I molecule. c anti-a CTL (anti-RT1^a) and c anti-R1 CTL (anti-RT1.A^a) generated in primary MLC were assayed on a panel of target cells (*a* and *b*). The same CTL populations were also assayed at one E/T ratio (10:1) on a, R1, and R8 targets in the presence of the A^a specific mAb R2/15S (*c* and *d*); control levels of lysis without antibody are shown by open symbols.

ecule, since lysis of all appropriate male targets was blocked completely by the mAb R2/15S (Fig. 4, *c* and *d*).

Parental a Cells Can Make an A^a-specific Cytotoxic Response Against Recombinant Stimulators. We next asked whether an antigenic difference between the two forms of RT1.A^a could be detected by direct immunization. Unprimed (c × a)F$_1$ lymphocytes were cultured with R1 stimulator cells in MLC, and assayed 5 d later for cytolytic activity. This responder/stimulator combination (A^{c}B^{c}D^{c}C^c × A^{a}B^{a}D^{a}C^a anti-A^{a}B^{c}D^{c}C^c) was chosen to exclude conventional incompatibilities, leaving just the

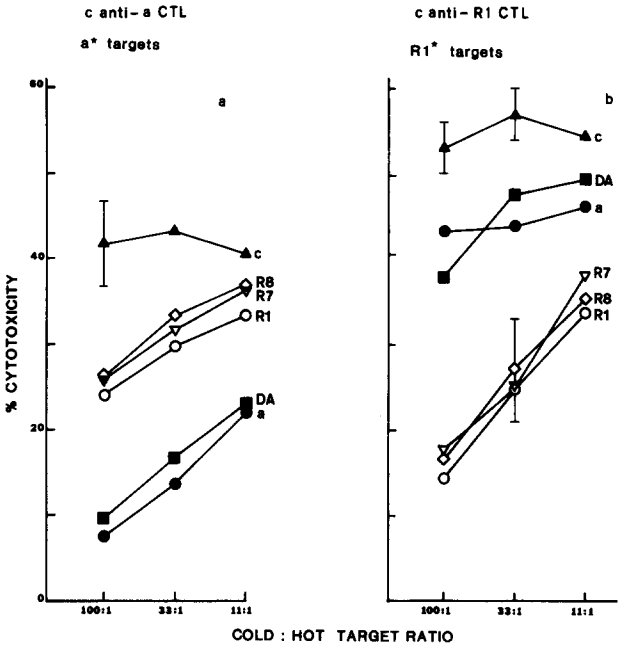

FIGURE 3. A^a specific alloreactive CTL can distinguish between parental (a) and recombinant (R1, R7, R8) cells in cold target competition assays. A panel of unlabeled (cold) target cells from various strains was used to compete for the lysis of ^{51}Cr-labeled a targets (a*) by c anti-a CTL (*a*), and of ^{51}Cr-labeled R1 targets (R1*) by c anti-R1 CTL (*b*). Both CTL populations were assayed at an E/T ratio of 50:1.

proposed difference between a and recombinant forms of the A^a class I molecule. CTL raised in this combination killed targets of all three recombinant strains (Fig. 5 *a*), and the lysis of R1 targets was completely blocked by R2/15S (Fig. 5 *b*), confirming the existence of a "recombinant-specific" determinant associated with the A^a class I molecule. R1, R7, and R8 cold targets all blocked the lysis of ^{51}Cr-labeled R1 targets by these CTL (Fig. 5 *c*), showing that the same antigenic determinant was expressed by cells for all three recombinant haplotypes. Responses of similar specificity were subsequently generated in the combinations (a × u)F_1 anti-R8 and (DA × c)F_1 anti-R1 and in the combination (DA × AO)F_1 anti-R8, where there are also minor histocompatibilities (data not shown). Targets of the responder parental strains (i.e., a and c in Fig. 5 *a*) were not killed, and (c × a)F_1 lymphocytes did not develop any cytolytic activity when cultured with a or c stimulators (data not shown), thus distinguishing the present findings from classical "F_1 antiparental" responses (26, 27).

The lack of appropriate recombinant strains made it impossible to generate CTL in combinations where determinants on the parental a specific form of the A^a molecule were the only known incompatibility. Weak CTL responses specific for these determinants were obtained, however, when unprimed R1 cells were stimulated with a cells (Fig. 6). While a small component of this response may have been directed against antigens encoded by the B^a, D^a, and C^a regions, most of it was directed against the A^a molecule since the lysis of a targets could be blocked effectively by the mAb R2/15S (Fig. 6 *b*). This result confirmed that there was a reciprocal antigenic difference between parental a and recombinant forms of the A^a class I mole-

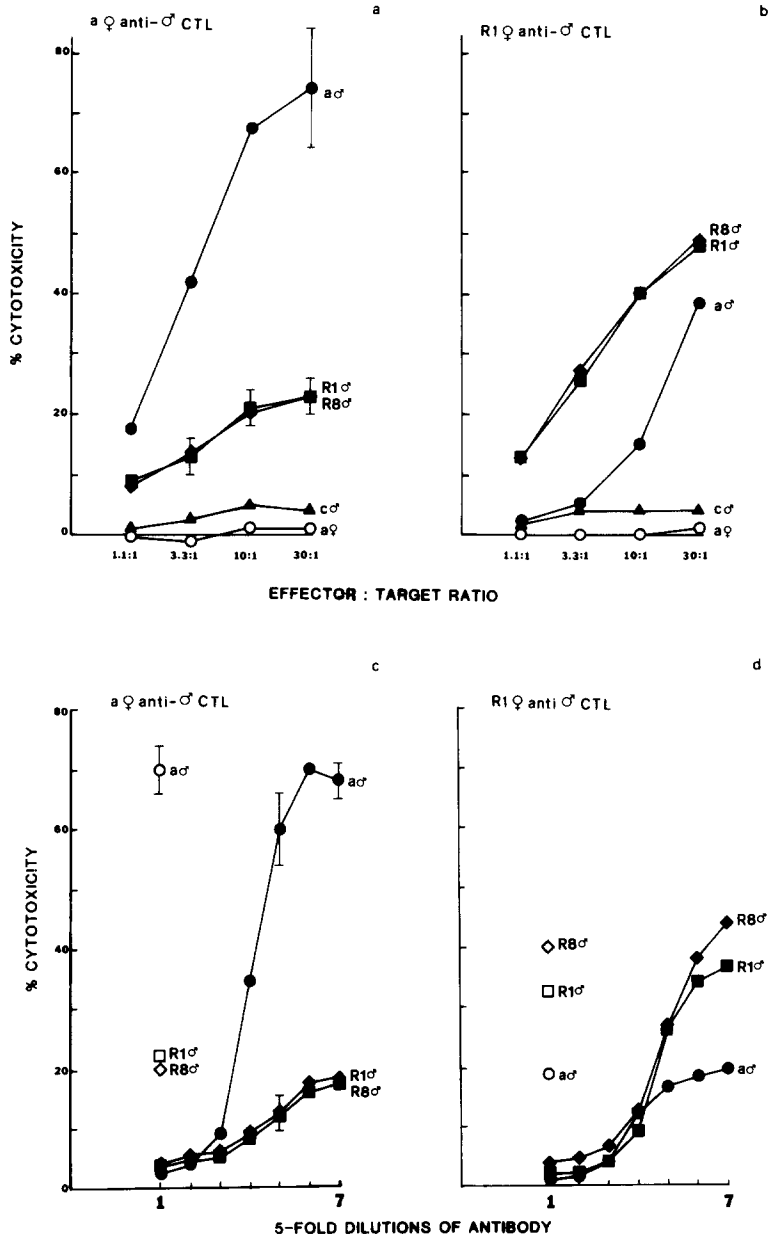

FIGURE 4. A^a-restricted, H-Y-specific CTL distinguish between parental (a) and recombinant (R1, R8) male targets. a and R1 female anti-male CTL were generated by immunisation with syngeneic male cells followed by an in vitro stimulation. CTL were assayed on a panel of male and female cells (*a* and *b*). The same CTL populations were assayed at one E/T ratio (30:1) on a, R1, and R8 male targets in the presence of the A^a-specific mAb R2/15S (*c* and *d*); control levels of lysis without antibody are shown by open symbols.

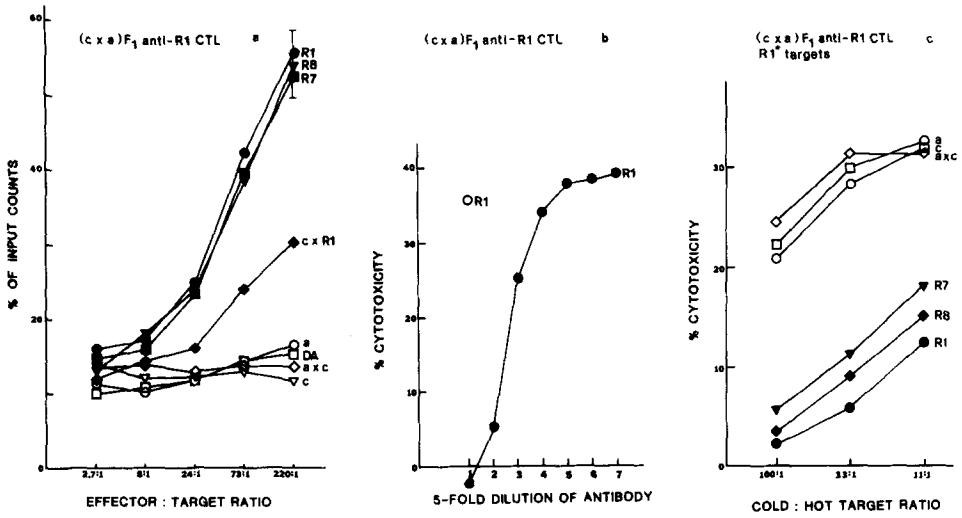

FIGURE 5. Cells carrying the parental RT1^a haplotype can make a primary cytotoxic response against the recombinant form of the A^a class I molecule. (c × a)F$_1$ anti-R1 CTL generated in primary MLC were assayed on a panel of target cells (*a*). Only targets carrying a recombinant haplotype (*filled symbols*) were killed. The ordinate in this panel exceptionally records lysis as percent of input counts for all targets due to a lost spontaneous release sample. CTL raised in the same combination were assayed at one E/T ratio (30:1) on R1 targets in the presence of the A^a-specific mAb R2/15S (*b*); the control level of lysis without antibody is shown by the open circle. (c × a)F$_1$ anti-R1 CTL were also assayed at one E/T ratio (18:1) on ^{51}Cr-labeled R1 targets in the presence of unlabeled (cold) target competitors of parental and recombinant genotype (*c*).

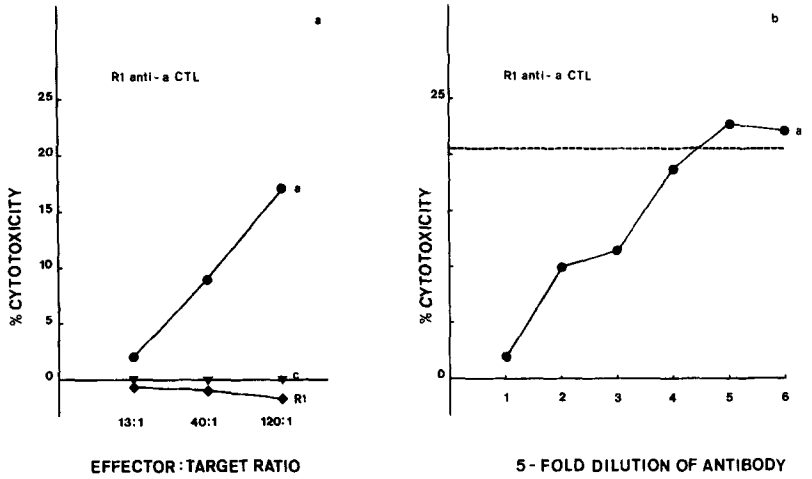

FIGURE 6. Recombinant cells can make a primary cytotoxic response against the parental form of the A^a class I molecule. R1 anti-a CTL generated in primary MLC were assayed on a panel of target cells (*a*). These CTL were also assayed at one E/T ratio (120:1) on parental a targets in the presence of the A^a-specific mAb R2/15S (*b*); the control level of lysis without antibody is shown by the dotted line.

cule. From now on, the parental a form of this molecule will be called A^{a+}, and the recombinant (R1, R7, R8) form A^{a-}. In a preliminary report (28) the two forms were called A^a and $A^{a'}$.

The Expression of A^{a+} Is Determined by an Interaction between the RT1.A^a Region and a trans-acting Gene Mapping to the RT1.B^a, RT1.D^a, or RT1.C^a Regions. Further experiments used cells from the (a × R1)F_1 hybrid to distinguish between *cis*- and *trans*-acting mechanisms responsible for the antigenic consequences of intra-MHC recombination. A *cis*-acting mechanism, such as an intra-genic recombinational hotspot (29), would result in these cells co-expressing heterozygous levels of A^{a+} and A^{a-}. In fact, (a × R1)F_1 cells were found to express no detectable A^{a-} and homozygous levels of A^{a+}, consistent with a dominant *trans*-acting mechanism. Fig. 7 shows the results of three different experimental approaches to ask whether (a × R1)F_1 cells expressed A^{a-}. These cells were tested as targets for (c × a)F_1 anti-R1 CTL (Fig. 7 *a*); as cold target competitors of the lysis of ^{51}Cr-labeled R1 targets by these CTL (Fig. 7 *b*); and for the ability to stimulate (c × a)F_1 responder cells in primary MLC (Fig. 7 *c*). No A^{a-} was detected on these cells in any of the assays. In contrast, each experiment showed that heterozygous levels of A^{a-} could be detected on (c × R1)F_1 cells, both as targets and as stimulators.

Evidence that (a × R1)F_1 cells expressed high levels of A^{a+} came from two different experiments. First, in cold target competition experiments of the kind shown in Fig. 3, (a × R1)F_1 cold targets were as effective as parental a cold targets at blocking the lysis of ^{51}Cr-labeled a targets by c anti-a CTL. (c × a)F_1 cold targets, which expressed heterozygous levels of A^{a+}, were considerably less effective competitors (data not shown).

The second line of evidence for the expression of homozygous levels of A^{a+} expression on (a × R1)F_1 cells came from the behavior of the mAb JY3/84. This antibody is one of a large number of mAbs raised against the A^a molecule (20). When tested for their binding to parental a and recombinant RBC, all these antibodies gave a higher binding level on a than on R1 or the other recombinants, reflecting a previously noted quantitative difference in antigen expression associated with the recombinant haplotypes (30, 31). In the case of JY3/84, however, binding to R1 was abnormally poor in comparison with parental a; indeed, it was scarcely detectable in some experiments. It appeared that JY3/84 detected a qualitative difference between the RT1.A^a molecules expressed on parental a and recombinant cells. We examined the binding of this antibody to erythrocytes from a panel of inbred strains and F_1 hybrids (Table II). (a × R1)F_1 cells bound about the same number of counts as parental a cells, which expressed homozygous levels of A^{a+}; this was considerably greater than the counts bound by (a × c)F_1 cells, which expressed heterozygous levels of A^{a+}. The amount of JY3/84 bound to R1 and (c × R1)F_1 cells was scarcely above background.

Demonstration of a trans-acting Modification of the RT1.A^a Class I Molecule by Complementation in F_1 Hybrids. The binding assay shown in Table II includes data from a number of F_1 hybrids made between R1 and various inbred strains. Erythrocytes from two of these F_1 hybrids, (l(AGUS) × R1)F_1 and (l(F344) × R1)F_1, bound about the same amount of JY3/84 as (a × c)F_1 erythrocytes, although JY3/84 did not bind to homozygous l(AGUS) or l(F344) cells. The reactivity of cells from (l × R1)F_1 donors with mAb JY3/84 suggested that these heterozygotes were expressing

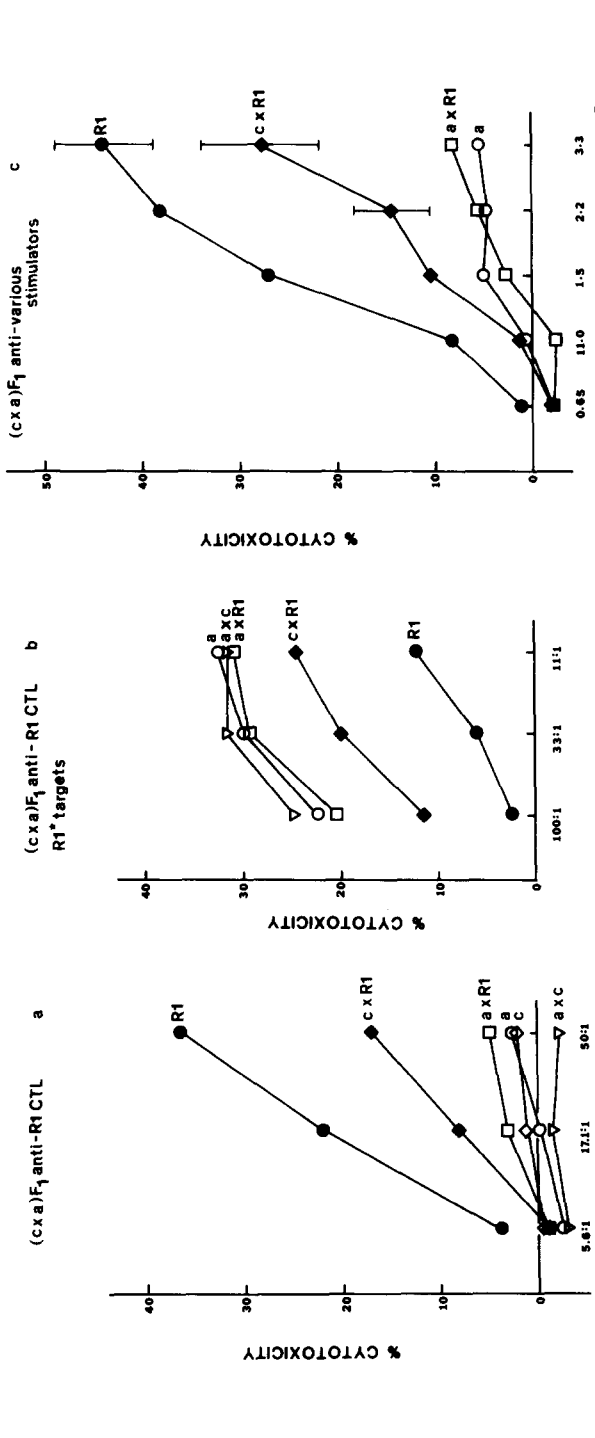

FIGURE 7. (a × R1)F_1 cells do not express detectable levels of A^{a-}. (c × a)F_1 anti-R1 CTL, specific for A^{a-}, were assayed on a panel of target cells (*a*). CTL raised in the same combination were assayed at one E/T ratio (18:1) on ^{51}Cr-labeled R1 targets in the presence of unlabeled (cold) target competitors (*b*). (c × a)F_1 cells were also stimulated in vitro with R1, (c × R1)F_1, (a × R1)F_1, or a cells, then assayed for cytotoxicity on R1 targets (*c*). In this last assay, varying numbers of responder cells were cultured in 96-well plates with irradiated stimulator cells of different genotype; 5 d later, 10^4 ^{51}Cr-labeled R1 cells were added directly to each well, and lysis determined after a further 4-h incubation period.

TABLE II
Binding of mAb JY3/84 to A^a on RBC from Various Rat Strains and Hybrids

Rat	Anti-Ig bound	Anti-Ig bound without JY3/84
	cpm	*cpm*
a*	1,659 (65)	67 (1)
R1	158 (19)	39 (9)
(a × R1)F_1	1,488 (428)	66 (12)
c	-‡	
(a × c)F_1	502 (28)	54 (20)
(c × R1)F	154 (19)	43 (2)
l(F344)	50 (9)	36 (10)
(l(F344) × R1)F_1	832 (78)	66 (11)
l(AGUS)	46 (7)	46 (9)
(1(AGUS) × R1)F_1	731 (18)	58 (7)

Data are means of triplicate determinations (+/- SD).
* All rats are congenic on the PVG background (see Table I).
‡ Not included in this experiment, but no binding of JY3/84 above background seen in several other experiments.

A^{a+} at heterozygous levels and not the expected A^{a-}. We tested this directly by using Con A blasts from these rats as targets for (c × a)F_1 anti-R1 CTL, specific for the A^{a-} form of the antigen. Fig. 8 shows that there was no significant lysis of these target cells; the control (c × R1)F_1 and (u × R1)F_1 targets were lysed quite effectively, showing that heterozygous levels of A^{a-} expression were detectable in this assay.

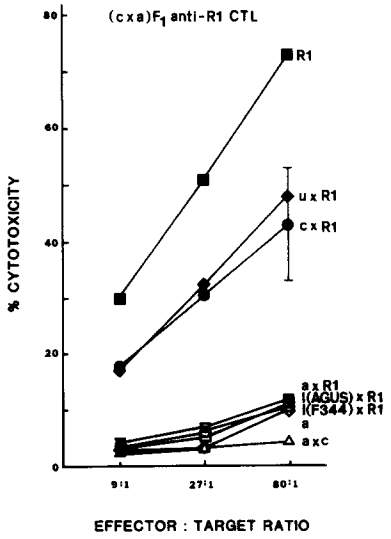

FIGURE 8. (l × R1)F_1 cells express no detectable A^{a-}. CTL specific for the A^{a-} form of the A^a class I molecule, generated in the combination (c × a)F_1 anti-R1, were assayed on a panel of target cells.

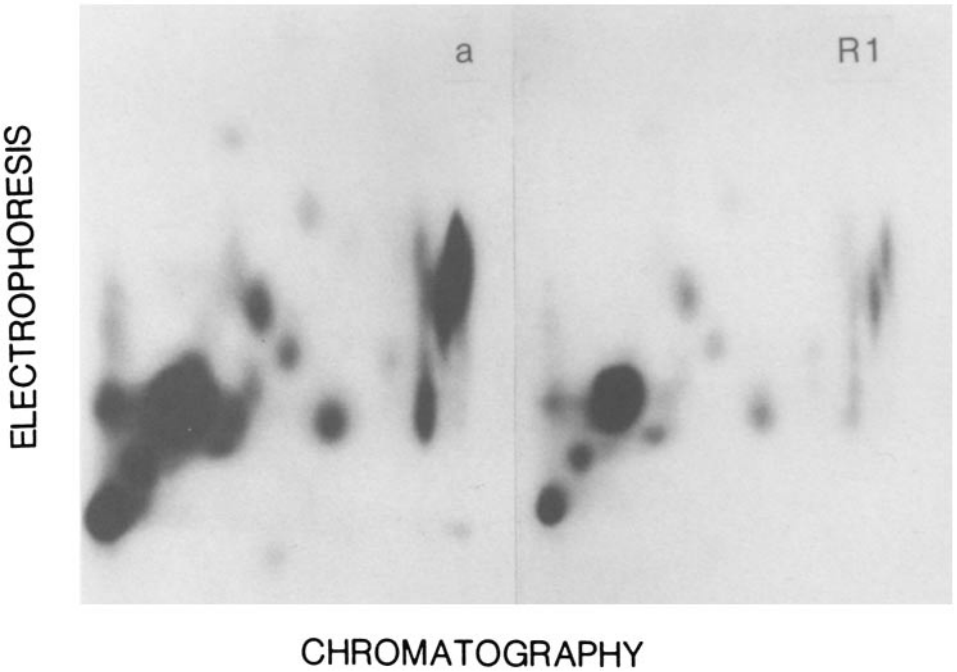

FIGURE 9. Two-dimensional peptic peptide maps of RT1.A^a H chains precipitated from a and R1 lymphoid cells by the RT1.A^a-specific mAb, R3/13. Tyrosine peptides were labeled with ^{125}I. Autoradiographic exposure, 10 d.

This experiment proved that the expression of A^{a-} was determined by a *trans*-interaction between the RT1.A^a region and the dominant allele of a locus, which, in the *a* haplotype, mapped to the right of the RT1.A/RT1.B recombination site(s). The dominant allele at this locus is carried by the *a* and *l* haplotypes, while a recessive (possibly null) allele is carried by the c and u haplotypes. We have named this locus *cim* (for *class I modification*), and suggest that the dominant allele be called *cim^a* and the recessive or null allele *cim^b*.

No Differences Observed between A^{a+} and A^{a-} on Two-dimensional Peptic Peptide Maps. A^a class I molecules were immunoprecipitated from detergent lysates of lactoperoxidase-iodinated a and R1 Con A blasts using the A^a-specific mAb R3/13 conjugated to Sepharose 4B. Two-dimensional peptic peptide maps were prepared of the heavy (46-kD) polypeptide as described elsewhere (22). No differential peptides were seen in several comparisons (Fig. 9); in contrast, RT1.A class I polypeptides from eight different major haplotypes tested showed striking differences (22).

Patterns of Biosynthetic Intermediates of A^a differ in a and R1 Cells. Although the previous analysis showed no difference in peptide structure between the parental and recombinant forms of A^a, examination of the biosynthesis of the antigen using pulse-chase studies of internally labeled Con A blasts clearly indicated a difference in the processing rates of the two forms of the antigen (Fig. 10). In parental a cells the increase in molecular weight corresponding to the fully mature A^{a+} antigen was observed to begin ~30 min post-pulse. All the labeled material had progressed into

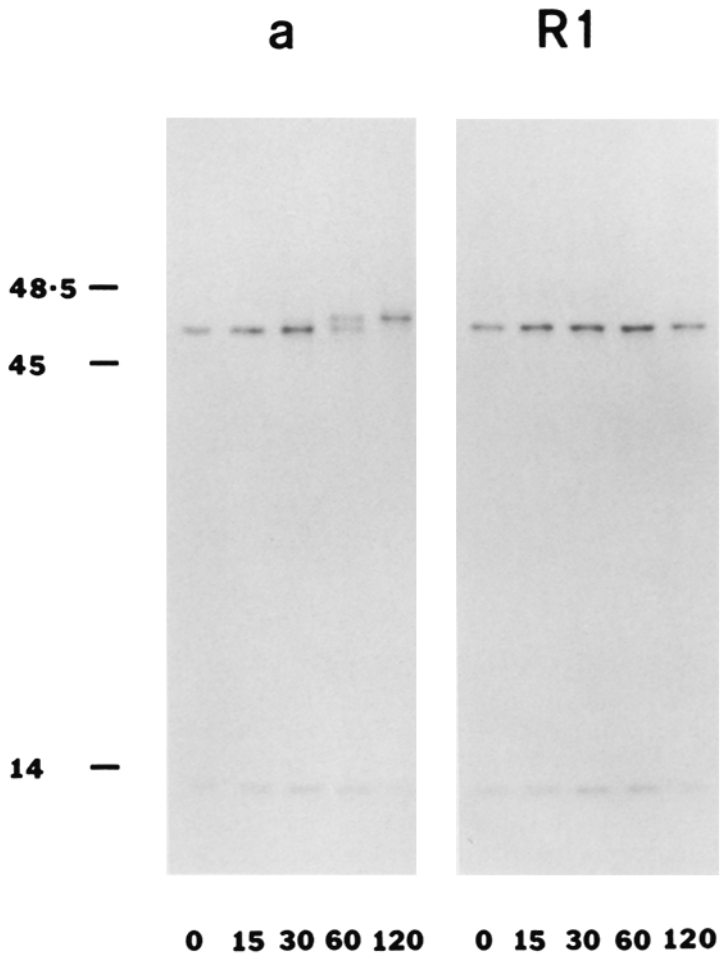

FIGURE 10. Pulse-chase experiment demonstrating biosynthesis of A^a in parental (a) and recombinant (R1) Con A blasts. After incorporation of ^{35}S-methionine and cold chase for the indicated times, lysed cells were immunoprecipitated with R3/13-Sepharose. Apparent molecular weights are kilodaltons, SDS-PAGE was performed on an 11% gel, and fluorography was performed for 7 d at −70°C.

the upper molecular weight form by 120 min. By contrast, in R1, the A^{a-} antigen remained in the lower form for a much greater length of time, with little indication of the upper form appearing even after 120 min. The pattern seen for the processing of A^{a+} corresponded well with that previously reported for both H-2 and HLA class I antigens (32–34).

Discussion

We describe in this paper the discovery of an MHC-linked locus, *cim*, that controls the structural modification of a rat class I major transplantation antigen. This

locus influences the antigenic structure of the RT1.A^a class I molecule (A^a), but maps outside the RT1.A region. It appears to have at least two alleles: a dominant allele, *cim*^{a}, and a recessive or null allele, *cim*^{b}, determining two alternative forms of the A^a molecule, A^{a+} and A^{a-}, respectively. From an immunological standpoint, the antigenic difference between the two forms is reminiscent of that seen between wild type and the stronger mutant forms of the mouse H-2K^b class I molecule, being readily demonstrated with both alloreactive CTL (including primary in vitro responses) and MHC-restricted CTL, but more difficult to detect serologically (35, 36). Experimental results presented elsewhere (37) may now be interpreted to suggest that antigenic differences controlled by this locus determine the rejection of lymphoid grafts, but so far there is no evidence for rejection of skin grafts (unpublished results). The structural basis for this antigenic difference is yet to be determined, but the genetic control of the system and preliminary biochemical data showing anomalous glycan processing in *cim*^{b} homozygotes exclude some possible explanations for the findings and suggest others.

It is unlikely that the antigenic difference between A^{a+} and A^{a-} is due to a difference in the primary sequence of the A^a product since the expression of A^{a+} can be complemented in *trans* by crossing a *cim*^{b} haplotype expressing A^{a-}, such as RT1^{r1} (see Table I), with the *cim*^{a} haplotype RT1^l, which does not itself contain the A^a allele. Thus, the A^a gene is expressed as A^{a+} or A^{a-} forms, depending only on the identity of the allele at the *cim* locus. Other interpretations, in which the *cim* locus product determines alternative splice forms of RT1.A^a, or conceivably even regulates the expression of similar but distinct structural genes coding for A^{a+} and A^{a-}, are constrained by the evidence presented in Fig. 9 showing identical peptide maps for A^{a+} and A^{a-}. Such interpretations are, however, now amenable to direct experimental test, since the RT1.A^a classical gene product has recently been cloned and expressed as a cDNA (Rada, C., R. Lorenzi, S. Powis, J. van den Bogaerde, P. Parham, and J. C. Howard, manuscript in preparation).

The antigenic difference between A^{a+} and A^{a-} is clearly connected in some way with the anomalous post-translational processing of the A^{a-} form of antigen. The increase in molecular weight of the A^{a+} product after a 30–45-min chase period is consistent with the typical biosynthetic behavior of H-2 and HLA class I molecules (32–34), and is attributed to the shift from high mannose to complex-type glycans, and to terminal sialylation during traffic through the Golgi (32–34). Recent evidence is consistent with this interpretation, and suggests that the persistent low molecular weight A^{a-} form retains the high mannose type glycan for a prolonged period (unpublished results). It may be relevant to this observation that the A^{a-} form of antigen is known to be relatively under expressed on the surface of resting lymphocytes and erythrocytes (30, 31).

It is possible that the *cim* product is an enzyme directly involved in glycan modification. There is already evidence for both glycosidase and glycosyl transferase activity mapping to the MHC. The best characterized of these loci is *Neu-1*, mapping in H-2 between E and D (38–40). This locus specifies a neuraminidase whose known action is to modify the processing of several enzymes, including a mannosidase expressed in the liver and in lymphocytes (40). An equivalent activity has been mapped to the rat MHC (41), but not yet located as to region. The critical question, however, is whether the differential post-translational modification of A^{a+} and A^{a-} is directly responsible for the different antigenic specificity the two forms present

at the cell surface. It must be emphasized that there is, as yet, no evidence for distinct glycan structures on the membrane forms of A^{a+} and A^{a-}. Furthermore, despite earlier claims (42, 43), it has been difficult to find either sugar-defined or sugar-modified T cell or antibody epitopes on classical class I molecules in several well-analyzed systems (44–46).

There is, however, a precedent involving the mouse nonclassical class I molecule, Qa-1, that may be relevant to the present findings. Qa-1 has been reported to occur in a number of allelic forms, as detected by CTL, some at least of which appear to be dependent on normal *N*-linked glycosylation (47). Furthermore, it has recently been shown that Qa-1, like A^a, is subject to structural modification by the product of a *trans*-dominant MHC-linked gene mapping to the H-2D region (48). It will therefore be of interest to know whether anomalous post-translational processing as described in the present report also occurs in the Qa-1 system.

If glycan modification by the *cim* system is indeed responsible for our own findings, it will be important to discover why, in this case, the effect on T cell recognition is so marked. Crystallographic data for the human classical class I molecule, HLA-A2, suggest that the single *N*-linked glycan on the first domain may be oriented away from the presumed peptide-binding and TCR interaction region (49), a result consistent with the general failure to find significant dependence of T cell specificity on glycans. RT1.A^a alone among known classical class I sequences has *N*-linked glycosylation signal sequences on the first and third domains (Rada, C., R. Lorenzi, S. Powis, J. van den Bogaerde, P. Parham, and J. C. Howard, manuscript in preparation). Conceivably this unusual arrangement of glycans could be subject to structural modification via the *cim* system and could in turn transmit a structural alteration to the peptide-binding cleft. Such an effect might be expected to have the kind of large scale antigenic consequences reported here.

We should finally consider the possibility that the primary action of cim is not on glycan modification as such, but rather on the ability of RTIA^a molecules to proceed normally through intracellular processing. The result of the pulse-chase study shown in Fig. 10 would be consistent with the possibility that the A^{a-} form is retained for an abnormally long time in the endoplasmic reticulum or early Golgi, while the presence of the dominant cim^a product releases this inhibition of transport. Recent experiments suggest that polymorphic sequences of the A^a molecule itself contribute to its sensitivity to the cim phenomemon, since classical class I molecules other than A^a from cim^b strains seem to be processed with normal kinetics (unpublished results). It is not clear how retention of RTIA^a in an early biosynthetic compartment might affect the antigenic activity of those molecules that do reach the membrane. However, since class I molecules may be loaded with endogenous peptides during assembly, the possibility that the cim product may be involved in some allele-specific way with the loading process is worth considering.

Mapping of the *cim* locus within the MHC is presently in progress. Preliminary results suggest that the locus maps between RT1A and RT1D (see Fig. 1) and it is therefore unlikely that the *cim* locus will prove to be identical to the H-2D region–linked Qa-1-modifying locus (48) referred to above. In view of the fact that the whole region homologous to the RT1A-RT1D interval has been cloned in the mouse (29) and much of it also cloned in the $RT1^a$ and $RT1^c$ haplotypes of the rat (50), it is likely that the molecular identity of the cim product will soon be known.

Summary

The RT1.A locus of the rat MHC encodes the H chain of the single classical class I molecule of this species. One of the alleles of this polymorphic locus, RT1.A^a, is present in several laboratory inbred, congenic, and MHC recombinant rat strains. Studies of the RT1.A^a class I molecule from a number of these strains as a target for CTL show that its antigenicity, both as an alloantigen and a restricting element, is subject to gain and loss alterations by the action of a gene mapping in the MHC to the right of RT1.A. This locus is apparently present in two allelic forms (one possibly a null allele) corresponding to the presence or absence of a dominant *trans*-acting modifier, and has been named *class I modification*, or *cim*. The antigenic change brought about by cim is scarcely detectable serologically but highly immunogenic for CTL. Biochemical investigations show that cim affects the post-translational modification of RT1.A^a.

We thank our colleagues Drs. Nick Holmes, Pat Jones, Peter Parham, and Prim Singh for critical reviews of the manuscript, and Julia Goddard, Joan Payne, and Jenny Richards for preparing the manuscript for publication.

Received for publication 24 October 1988 and in revised form 27 March 1989.

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