Control of Rat Natural Killer Cell-mediated Allorecognition by a Major Histocompatibility Complex Region Encoding Nonclassical Class I Antigens

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Summary

The ability of natural killer (NK) cells to eliminate normal allogeneic hemic cells is well established in several species including mice, rats, and humans. The controlling elements for NK susceptibility in these species map to the major histocompatibility complex (MHC), but in contrast to findings in mice and humans, the mode of inheritance is not always recessive in rats. This finding is not easily explained by the missing self and hemopoietic histocompatibility (Hh) models for NK recognition, and has led to the idea that certain alloantigens may trigger NK cell reactivity. In our in vitro system for assessing rat NK alloreactivity, we have employed target and inhibitor cells from a large panel of MHC congenic, intra-MHC recombinant and MHC mutant rat strains, as well as appropriate F1 hybrids between them, and we show the following: (a) The nonclassical class I (RT1.C) region was most important in determining the susceptibility of target cells to alloreactive NK cells in vitro. Lymphocyte susceptibility to lysis in vivo also mapped to the C region, which supports the concept that the in vivo and in vitro alloreactivity assays reflect the same recognition process. (b) Four different RT1-controlled NK allospecificities (represented by the u, i, a, and n haplotypes) could be discerned when we used polyclonal NK cells from the PVG (RT1') strain as effector cells. Three of the target specificities recognized were controlled mainly by the RT1.C region. (c) The expression of RT1.C region-controlled parental strain NK allodeterminants could be demonstrated in F1 hybrids heterozygous for the C region alone and were therefore inherited nonrecessively. (d) Loss of an RT1.C region-controlled NK allospecificity could be shown with the MHC mutant LEW.1LM1 rat strain characterized by a genomic deletion of about 100 kb of the C region. Taken together, these observations have demonstrated a major importance of the nonclassical class I region, i.e., RT1.C, in controlling rat NK allorecognition, and have thereby assigned a hitherto undescribed immunological property to this region. Furthermore, some of the present data are consistent with the existence of polymorphic NK-triggering alloantigens that are coded for by the RT1.C region.

NK cells were originally defined by their ability to lyse spontaneously certain neoplastic cells in vitro. The reactivity was considered not to be MHC restricted, but an MHC-linked influence on NK activity was nevertheless observed (1). Later studies showed that NK cells could eliminate lymphoma cells that lacked expression of self MHC class I molecules (2), supporting the missing self hypothesis for NK recognition postulated by Kärre (3) and Ljunggren and Kärre (4). This hypothesis has lately received strong support by studies of MHC class I transfected target cells (5, 6), and MHC class I transgenic (7-9) and β2 microglobulin knockout mice (9-11).

The characterization of NK cell reactivity was examined independently in mouse studies of the resistance against parental and allogeneic bone marrow cell (BMC)1 grafts (12,

1 Abbreviations used in this paper: ALC, allogeneic lymphocyte cytotoxicity; BMC, bone marrow cell; CHO, Chinese hamster ovary; Hh, hemopoietic histocompatibility; PEC, peritoneal exudate cell; RT1, MHC of the rat.
13). Here, a new set of recessively inherited hemopoietic histocompatibility (Hh) antigens had been postulated (14, 15). The immunogenetic specificity and the Hh restriction of the effector cells served as distinguishing features towards NK activity in vitro, however (16, 17). The major Hh-1 antigens are controlled by genes closely linked to, but not identical to H-2D (18). The recently demonstrated role for MHC class I molecules as specificity-determining elements in different NK recognition models in mice and humans, e.g., that of H-2D in a transgenic mouse model (7, 8), has prompted a revision of the Hh recognition model and suggests that the MHC class I molecule itself or peptides derived from it, forms an integrated part of the Hh antigen (19, 20).

Also, allogeneic lymphocyte grafts are subject to prompt elimination in unsensitized recipients (21, 22). This phenomenon is termed allogeneic lymphocyte cytotoxicity (ALC) (21), and several lines of evidence strongly suggest that ALC is mediated by NK cells (21-24). Studies on the mechanisms underlying ALC have been facilitated by the development of an in vitro cytotoxicity assay (25, 26), demonstrating the ability of rat NK cells to mediate alloreactivity (27). The involvement of the MHC in ALC was shown with MHC congenic rats (28), and further studies with intra-MHC recombinant strains have suggested that the controlling elements map to the RT1.B/D (class II)-RT1.C (nonclassical class I) regions and not the classical class I (RT1.A) region in the rat (29).

The use of target and inhibitor cells from an MHC congenic rat strain, as well as backcrossed rats in the in vitro assay provided evidence that not only was susceptibility to NK cells controlled by the MHC, but also that there was evidence of specificity in the interaction between NK cells and targets of different allo-MHC types (27). This indicates a functional diversification of rat NK cells into subgroups with different allospecificities, as has been demonstrated for mouse NK cells with regard to H-2 recognition (20, 30) and with human allospecific NK cell clones (31-33). Target cell susceptibility or specificity was inherited in a nonrecessive fashion, however (27), in contrast to findings in mice and humans (14, 15, 20, 32-34). This observation was in line with earlier findings in rats where a positive alloantigen recognition model for ALC had been suggested (21, 35). It is therefore conceivable that MHC alleles may have both triggering and inhibitory effects on rat NK cells (36).

This study represents a detailed investigation of the role of the MHC in controlling rat NK allorecognition in vitro. The results support the concept that certain allogeneic MHC alleles may activate rather than inhibit rat NK cell lysis and that the relevant polymorphic target cell alldeterminants are controlled by the nonclassical class I region, i.e., RT1.C, in this species.

Materials and Methods

Animals. Breeding pairs from rat strains on PVG background (PVG, PVG.1U, PVG.1AV1, PVG.R8, and PVG mu/mu), and the AO strain were obtained from Harlan Olac (Bicester, UK), and the LOU/C and LEW strains from Charles River (Sulzfeld, Germany). They were reared under conventional conditions in Oslo and routinely screened for common rat pathogens. Other rats on LEW background were either maintained at the Zentralinstitut für Versuchstierzucht (LEW.1U, LEW.1A, LEW.1AV1, LEW.1N, LEW.1C, LEW.1AR2, LEW.1WR1, and LEW.1WR2) or at the Medizinische Hochschule (LEW.1R14, LEW.1R15, LEW.1LM1, and LEW.1LM2). The F1 hybrid crosses (LEW.1AR2 × LEW.1A and LEW.1U × LEW.1WR1) were bred in Oslo. The genetic constitution of the various rat strains used in this study is given in Table 1. The experimental protocol was approved by the institute veterinarian and registered by the Experimental Animal Board under the Ministry of Agriculture of Norway.

In Vitro Cytotoxicity Assay. The 4-h 1Cr-release assay for measuring rat NK alloreactivity in vitro has been described in detail previously (26, 27), but some modifications were made. Previous studies were based on the use of allogeneic lymphocytes (26, 27) or BMC (25, 27) as target cells, and it was necessary to include PHA during the cytotoxicity assay to obtain efficient lysis of allogeneic cells. In this study, Con A-activated T cell blasts were used as targets. These were efficiently lysed by allogeneic NK cells in the absence of PHA.

In short, 5-10 × 10⁸ target cells were incubated with 3.7 MBq 3HCr/ml, washed three times, and 10⁶ cells were added to the effector cells (10⁴-10⁵ cells/well) in U-bottomed 96-well plates. In the cold target inhibition experiments, effector cells (10⁶ cells/well) were added first, followed by inhibitor cells before target cells (10⁴ cells/well). The cells were mixed and the microplates centrifuged for 1 min at 300 g before incubation for 4 h at 37°C in 5% CO₂/humidified air. Supernatants were harvested with a Titertek harvesting system (Skatron, Lierbyen, Norway). The percent specific lysis was calculated as described (26). Spontaneous release was usually 10-25%. The results are presented as median values from triplicates for each effector/target or target/inhibitor (T/I) cell ratio.

Preparation of Target Cells. Con A-activated T cell blasts were generated from spleen cells separated on Lymphoprep, density = 1.077 g/ml (30 min, 400 g; Nycomed Pharma, Oslo, Norway). The mononuclear cells were adjusted to 2 × 10⁶ cells/ml and cultured for 2-4 d in RPMI 1640 supplemented with 5% FCS and 5% normal rat serum (both heat inactivated), 2 mM L-glutamine, 1 mM Na pyruvate, 5 × 10⁻³ M 2-ME, and 5 μg/ml of Con A. Before use as targets or inhibitors, dead cells were removed by Lymphoprep centrifugation. In some experiments, BMC were used as targets. BM was flushed out of tibias and femurs, and the cells suspended and filtered through a thin layer of cotton wool before separation on Lymphoprep.

Preparation of Effector Cells. Two new methods, to be described in detail elsewhere, were employed to generate large numbers of IL-2 activated NK cells. In the first (37, and Lewik, G., J. T. Vaage, C. Naper, H. B. Benestad, and B. Rolstad, manuscript submitted for publication), NK cells were retrieved from the peritoneal cavity of rats implanted 7-10 d earlier with cell-impermeable diffusion chambers containing cultures of transfected Chinese hamster ovary (CHO) cells that secrete rat IL-2 (38). The number of peritoneal exudate cells (PEC) recovered, varied between 100 and 1,000 × 10⁶ cells per rat after Lymphoprep centrifugation. About 50% of the cells were NK cells as judged by LGL morphology and flow cytometry after staining with the 3.2.3 mAb reacting with the NK-R1 molecle (39), expressed at high density on NK cells. The main contaminants were macrophages staining with OX41 and lymphocytes. The percentage of NK cells was higher (70-80%) among PEC from athymic mu/mu rats; here negligible numbers of cells expressed T cell markers. In the second method (Naper, C., unpublished observations) spleen...
cells were depleted of CD3+ cells, and NKR-P1+ cells were positively selected with magnetic Dynabeads (a kind gift from Dynal, Oslo, Norway) coated with the 3.2.3 mAb. The cells were then cultured for 1-3 wk (5% CO2/humidified air at 37° C) in RPMI 1640 (without Hepes) supplemented with 10% FCS, 2 mM glutamine, 1 mM Na pyruvate, 5 × 10^-5 M 2-ME, and rat rIL-2 (at ~1,000 IU/ml as compared with the activity of human rIL-2) obtained from the cell culture supernatant of the transfected CHO cell line (see above). The cells released the beads after the first few days in culture and were shown to consist entirely of CD3-, CD5-, TCR-a/b-, 3.2.3+ NK cells. In some experiments we used unstimulated nylon wool nonadherent spleen cells from mu/mu rats that we have characterized previously (27).

Measurement of ALC In Vivo. Lymphocytes, obtained by gentle disruption of cervical and mesenteric LN followed by vigorous pipetting, were filtered through a thin layer of cotton wool and labeled at 5-10 × 10^6 cells/ml with 0.4 MBq 51Cr/ml. The cells were washed three times and 15 × 10^6 cells were injected intravenously. After 20 h, the radioactivity associated with cervical and mesenteric LN was counted. 51Cr is retained in viable lymphocytes that enter the different LN. If the injected lymphocytes are eliminated, the isotope is released to the tissue fluids and subsequently excreted by the kidneys. The rapid rejection of lymphocytes is therefore measured as decreased radioactivity levels in the LN of allogeneic compared with that of syngeneic recipients, i.e., the LN index, which is the most reliable and sensitive parameter of cell destruction in vivo (21, 40).

Statistics. Statistical significance was evaluated with a two-sided nonparametric Wilcoxon-van Elteren test (41). Triplicate values at each effector/target or target/inhibitor cell ratio were included in the analyses.

Results

PVG-mu/mu NK Cells Discriminate between Four Different RT1-controlled Target Specificities. The ability of a polyclonal NK cell population from PVG mu/mu (RTI") rats to discriminate between different MHC congenic target cells, was tested in cold target inhibition experiments. Lysis of the MHC incompatible LEW (RTI), LEW.1N (RTI), LEW.1A (RTI), and LEW.1U (RTI) Con A blasts was inhibited about equally well and in a cell dose-dependent manner with inhibitor cells syngeneic with the labeled target cells (Fig. 1, A-D). The inhibition caused by LEW.1C (RTI') lymphoblasts, mismatched with the effector cells only in the non-MHC genetic background, was minimal. The degree of inhibition with MHC third party inhibitor cells varied depending on the labeled target cell studied. Only slight inhibition of lysis of RTI" Con A blasts was obtained with unlabeled third party RTI", RTI', or RTI" blasts (Fig. 1 D) and the specific component in the recognition of RTI" targets (the mu NK allospecificity) was therefore marked. The l NK allospecificity was less pronounced since unlabeled RTI", RTI", or RTI" Con A blasts cross-inhibited the lysis of RTI" cells substantially (Fig. 1 A). The n allospecificity

![Image](https://via.placeholder.com/150)

Figure 1. NK cells display four different RTI-controlled allospecificities in cross-competition experiments. As effector cells were used NK cells from the peritoneal cavity of PVG-mu/mu (RTI") rats implanted with diffusion chambers containing transfected CHO cells secreting rat rIL-2. Target and inhibitor cells were Con A-activated T cell blasts from MHC congenic rats: LEW (RTI"), LEW.1N (RTI), LEW.1A (RTI"), and LEW.1U (RTI") Con A blasts cross-inhibited the lysis of RTI" cells substantially (Fig. 1 A). The n allospecificity

### Table 1. The Genetic Constitution of the Inbred, MHC Congenic, Recombinant, and Mutant Rat Strains Used

| Strain   | RTI haplotype | RTI regions | Non-MHC background |
|----------|---------------|-------------|-------------------|
| LEW      | l             | A           | LEW               |
| LEW.1U   | u             | u           | LEW               |
| LEW.1A   | a             | a           | LEW               |
| LEW.1AV1 | av            | a           | LEW               |
| LEW.1N   | n             | n           | LEW               |
| LEW.1C   | c             | c           | LEW               |
| LEW.1AR2 | r3            | a           | LEW               |
| LEW.1WR1 | r4            | u           | LEW               |
| LEW.1WR2 | r6            | u           | LEW               |
| LEW.1R14 | r14           | u           | LEW               |
| LEW.1R15 | r15           | l           | LEW               |
| LEW.1LM1 | lm1           | l           | LEW               |
| LEW.1LM2 | lm2           | l           | LEW               |
| PVG      | c             | c           | PVG               |
| PVG mu/mu| c             | c           | mu/mu             |
| PVG.1U   | u             | u           | PVG               |
| PVG.1AV1 | av1           | a           | PVG               |
| PVG.R8   | r8            | a           | PVG               |
| AO       | u             | u           | AO                |
| LOU/C    | u             | u           | LOU/C             |

* Recombination point
was in itself no separate entity because the target specificity recognized was also expressed in the a haplotype, i.e., RT1\(^a\) lymphoblasts were as efficient as RT1\(^a\) cells in inhibiting lysis of RT1\(^a\) target cells (Fig. 1 B). This was not the case in the opposite direction, since RT1\(^a\) cells inhibited killing of RT1\(^a\) targets less well than did unlabeled RT1\(^a\) blasts (Fig. 1 C). It follows that two different target specificities were encoded by the a haplotype: one expressed only in the a haplotype and one expressed also in the n haplotype. Thus, four different RT1\(^a\)-controlled NK allospecificities could be discerned in cross-competition studies, viz., a\(^+\), a\(^{+n}\), I\(^+\), and u\(^+\).

**NK Susceptibility of Con A-activated T Cell Blasts In Vitro and Allogeneic Lymphocytes In Vivo Is Mainly Controlled by the RT1.C Region.** PVG.1U (RT1\(^{au}\)) NK cells efficiently lysed RT1\(^{au}\) (LEW.1A), but not RT1\(^{au}\) (LEW.1U) Con A blasts (Fig. 2 A). The role of the different RT1 subregions in controlling the susceptibility to lysis by PVG.1U NK cells was examined with lymphoblasts from the different a-u recombinants. Efficient lysis of Con A blasts was only observed with RT1.C and not with A and B/D region disparities. Thus, LEW.1WR1 (uua) and LEW.1WR2 (uaa), but not LEW.1AR2 (auu) and PVG.8 (auu) Con A blasts were efficiently killed by PVG.1U NK cells (Fig. 2 A). When PVG.1AV1 (RT1\(^{av1}\)) NK cells were employed, lysis of uaa but not aaa Con A blasts was seen (Fig. 2 B). Again the a-u recombinants mapped the controlling elements to the RT1.C region; uaa and auu but not uaa and uaa Con A blasts were lysed (Fig. 2 B). Although the a and av1 haplotypes differ in the RT1.C region, the two haplotypes encode similar target cell specificities as defined by RT1\(^a\) NK cells in cross-competition studies (data not shown), and it was therefore not surprising that RT1\(^a\) Con A blasts were spared from lysis by NK cells of the av1 haplotype.

In addition to the strong influence of RT1.C, some influence was also observed from the RT1.A region. In four experiments, RT1.C region compatible, but A region disparate PVG.R8 (auu) and LEW.1AR2 (auu) lymphoblasts were killed slightly better than fully MHC compatible uaa cells by PVG.1U NK cells (\(p < 0.001\) for PVG.R8 vs. LEW.1U at each E/T ratio). Conversely, A region–compatible, C region–incompatible LEW.1WR2 (uua) and LEW.1WR1 (uua) Con A blasts were slightly less sensitive to lysis than uaa cells (\(p < 0.01\) for LEW.1WR2 vs. LEW.1A) (Fig. 2 A). It is therefore possible that, in certain strain combinations, the NK susceptibility conferred to the target cells by the C region may be slightly modified by other RT1 subregions. Alternatively, the level of killing reflects the accumulated effects of separate effector-target combinations controlled in separate MHC regions.

LN cells from the different a-u intra-MHC recombinant and parental strains were labeled with \(^{3}^{1}^{1}Cr\) and injected intravenously into PVG.1U, AO, and LOU/C rats (all RT1\(^a\)). Analogous to the situation in vitro, RT1.C region–incompatible lymphocytes were rejected in vivo, whereas A or B/D region incompatibilities appeared to be of minor importance (Table 2). This supports the view that the same recognition process is measured in both assay systems.

**Mapping of Three RT1-controlled Target Specificities to the RT1.C Region.** The controlling elements for the expression of the NK-defined u, a, and l target specificities (see Fig. 1) were mapped with two different sets of intra-MHC recombinants and the respective parental strains in cross-competition studies. Experiments with inhibitor cells from both the l-u and the a-u recombinants mapped the controlling elements for the u NK allospecificity to the RT1.C and not to the A or B/D regions. Only unlabeled C lymphoblasts were efficiently inhibited by the lysis of RT1\(^a\) target cells (Fig. 3, A and C). The C mapping of the u allospecificity could also be demonstrated with BMC as target and inhibitor cells and was thus not dependent on activation antigens present on target/inhibitor cells that were specifically induced by Con A (data not shown).

The expression of the NK-defined l and a target specificities was similarly found to be controlled mainly by the RT1.C region with the relevant recombinant inhibitor cells. Thus, the most efficient inhibition of RT1\(^a\)-target cells was observed with uul and not with luu inhibitors (Fig. 3 B), and analogous results were noted for the a NK allospecificity (Fig. 3 D). Indications that the RT1.A/B/D regions influenced both target specificities were obtained, however. The inhibitory performance of uul lymphoblasts was slightly inferior to that of luu cells whereas luu lymphoblasts inhibited slightly better than uul cells (Fig. 3 B). A similar tendency was noted for the a target specificity, with the reservation that the discrimination was less well marked in this series of experiments (Fig. 3 D), in analogy with the results obtained when the same set of a-u recombinants was used to map the ele-
Table 2. Allogeneic Lymphocyte Cytotoxicity In Vivo Is Predominantly Controlled by the RT1.C Region

| Recipient Strain | RT1 haplotype | Donor Strain | RT1 haplotype | RT1 regions | LN index |
|------------------|---------------|--------------|---------------|-------------|---------|
| PVG.1U           | u             | LEW.1A       | a             | a a a a a | 0.093   |
| PVG.1U           | u             | LEW.1WR2     | r6            | u a a a a | 0.17    |
| PVG.1U           | u             | LEW.1WR1     | r4            | u u a a a | 0.14    |
| PVG.1U           | u             | LEW.1AR2     | r3            | a a a a a | 0.54    |
| PVG.1U           | u             | PVG.8        | r8            | a u u u u | 0.91    |
| AO               | u             | LEW.1WR2     | r6            | u a a a a | 0.086   |
| AO               | u             | LEW.1WR1     | r4            | u u a a a | 0.11    |
| AO               | u             | LEW.1AR2     | r3            | a a a a u | 0.37    |
| AO               | u             | PVG.8        | r8            | a u u u u | 0.77    |
| AO               | u             | LEW.1U       | u u u u u u u | 0.66    |
| LOU/C            | u             | LEW.1WR2     | r6            | u a a a a | 0.10    |
| LOU/C            | u             | LEW.1WR1     | r4            | u u a a a | 0.18    |
| LOU/C            | u             | LEW.1AR2     | r3            | a a a a u | 0.63    |
| LOU/C            | u             | PVG.8        | r8            | a u u u u | 1.2     |
| LOU/C            | u             | LEW.1U       | u u u u u u u | 1.3     |

ALC was calculated as the uptake of intravenously injected ¹⁵⁵Cr-labeled lymphocytes in cervical and mesenteric LN of allogeneic relative to syngeneic recipients ca. 20 h after i.v. injection (28). This referred to as the LN index and a low index means that the injected cells had been eliminated.

* No. of recipient rats injected.

Figure 3. NK-defined target cell specificities are controlled predominantly by the RT1.C region. Effector cells were as in Fig. 1. Target and inhibitor cells were Con A blasts from two different panels of intra-MHC recombinant strains and from the respective parental strains: (A and B) LEW.1U (Δ), LEW.1R15 (▼), LEW.1R14 (●), LEW (○), and LEW.1C (▲). (C and D) LEW.1U (Δ), PVG.8 (▼), LEW.1AR2 (▼), LEW.1WR1 (○), LEW.1WR2 (●), LEW.1A (□), and LEW.1C (▲). Labeled target cells are denoted above each graph. Results are presented as medians of three (A and B) or two (C and D) experiments in which the reciprocal specificities were measured at the same time. The C region mapping of the u NK allospecificity could also be demonstrated with BMC as target and inhibitor cells under the conditions previously described (27).
Nonrecessive Inheritance of RT1.C Region-controlled Target Cell Susceptibility and NK-defined Target Specificities. NK cells were generated from RT1<sup>+</sup> (PVG.1U, AO, and LOU/C), RT1<sup>−/−</sup> (PVG.1AV1) and RT1<sup>−</sup> (PVG mnu/mnu) strains. Target and inhibitor cells were from two different crosses designed to generate F₁ hybrids with heterozygosity only in the RT1.C region, i.e., (LEW.1U x LEW.1WR1)F₁ (uuu/a) and (LEW.1AR2 x LEW.1A)F₁ (aua/a) hybrids. In accordance with the previous data, PVG.1U, AO, and LOU/C NK cells lysed RT1.C region disparate LEW.1A (aaa) and LEW.1WR1 (uuu) Con A blasts, but not C region-compatible LEW.1AR2 (aua) and LEW.1U (uuu) lymphoblasts (Fig. 4, A and B). Lymphoblasts from RT1.C region heterozygous F₁ hybrids were also susceptible to lysis, though at an intermediate level when compared with that of the susceptible and resistant parental cells (Fig. 4, A and B). This was seen regardless of whether additional disparities between effector and target cells in the RT1.A-B/D regions were present (Fig. 4 B) or absent (Fig. 4 A).

Analogous results were obtained with RT1<sup>−/−</sup> NK cells recognizing allodeterminant(s) coded for by C<sup>a</sup> genes. PVG.1AV1 NK cells lysed the two different F₁ hybrid lymphoblasts more poorly than, and better than, the lysis of the respective sensitive and resistant parental strain cells (Fig. 4, C and D). Also here, additional incompatibilities at the RT1.A-B/D regions were found not to influence the nonrecessive inheritance pattern of the RT1.C region controlled target cell allodeterminant(s) (compare Fig. 4, C and D).

Lymphoblasts from the LEW.1AR2 (aua) and LEW.1A (aaa) strains expressed different NK-defined target specificities as detected by their relative inability to effectively cross-compete for the RT1<sup>+</sup> effector cells in cold target inhibition experiments (Fig. 5, A and B). The (LEW.1AR2 x LEW.1A)F₁ hybrid (aua/a) lymphoblasts expressed both parental target specificities, as shown by the intermediate inhibitory performance compared with the respective parental cells (Fig. 5, A and B). Some specificity was also seen when inhibition of lysis of the F₁ cells was measured, as expected, since the F₁ cells expressed both parental target specificities (Fig. 5 C). Loss of an NK-defined Target Specificity in an RT1.C Region Class 1-deletion Mutant Strain (LEW.1LM1). The importance of the RT1.C region in controlling rat NK allorecognition prompted us to study two mutant rat strains with deletions within this genetic region. The LEW.1LM1 (RT1<sup>−/−</sup>) strain, derived from the LEW strain, is characterized by a genomic deletion of 100 kb of class I cross-hybridizing fragments (42), which includes loss of a serologically defined polymorphic

Figure 4. Demonstration of nonrecessive inheritance of RT1.C region-controlled NK susceptibility. Effector cells were generated as in Fig. 1, but were from the strains denoted above each graph. Con A blasts were used as target cells: (A and C) LEW.1WR1 (□), LEW.1U x LEW.1WR1 (□, △), and LEW.1U (△). (B and D) LEW.1A (■), (LEW.1AR2 x LEW.1A)F₁ (●), and LEW.1AR2 (▲). Representative experiments are shown.
Figure 5. Demonstration of nonrecessive inheritance of parental NK-defined target specificities controlled by the RT1.C region. Effector cells were as in Fig. 1. Con A blasts were used as target and inhibitor cells: LEW.1AR2 (Δ), LEW.1A (□), (LEW.1AR2 × LEW.1A)F1 (○), and PVG (△). Labeled target cells are denoted above each graph. The results are medians from two experiments.

class I molecule (43, 44). Mutant LEW.1LM1 Con A blasts were killed by allogeneic PVG rnu/rnu and AO NK cells, but the cytotoxic level appeared to be somewhat lower than with the parental LEW lymphoblasts (Table 3). When tested in cross-competition experiments, it was found that the NK-defined I target specificity was lost in the LEW.1LM1 mutant. LEW.1LM1 cells inhibited the lysis of LEW cells no better than the MHC third party LEW.1U cells (Fig. 6 A). In contrast, the inhibitory capacities of LEW.1LM1 and LEW cells towards the unrelated u NK allospecificity were similar (Fig. 6 B). Finally, LEW cells inhibited the lysis of LEW.1LM1 cells equally well as did LEW.1LM1 themselves (Fig. 6 C). This showed that no new NK-defined target specificity, that was not expressed in the LEW strain, could be detected in the mutant. The deletion in LEW.1LM1 therefore appeared to affect the expression of the I target specificity rather selectively.

The LEW.1LM2 (RT1lm2) mutant strain is derived from the LEW.1LV3 (RT14) and LEW.1U (RT1u) strains and is the result of unequal crossing over in the C region resulting in a relatively small deletion compared with that of LEW.1LM1 (45). The RT1A-B/D regions of LEW.1LM2 are derived from LEW.1LV3 whereas most of the RT1C region is from the LEW.1U strain, and it was thus important to examine whether the C region-controlled u target specificity was expressed in the LEW.1LM2 strain. LEW.1LM2 Con A blasts were tested in cold target inhibition studies and were found to inhibit the lysis of RT1+ targets as efficiently as did RT1+ inhibitors themselves (Table 4). The relevant gene(s) coding for the u target cell allodeterminant was thus not deleted in LEW.1LM2.

**Discussion**

We have shown that the nonclassical class I region, i.e., RT1.C, plays a central role in controlling NK alloreactivity
in the rat. This is a new and unexpected finding, since apart from induction of alloimmune responses, no clear immunological functions have so far been assigned to this region. However, involvement of the RT1.C region is consistent with the pivotal role of class I molecules in other NK allorecognition models, both in mice (9, 17, 20) and in humans (33).

The present investigation demonstrated that four different MHC-controlled NK allospecificities could be discerned in cross-competition studies in vitro, corroborating and substantially extending one of our previous studies (27). Further, the use of intra-MHC recombinants showed that three of the NK allospecificities mapped primarily to the RT1.C region at the target cell level. The data imply that the MHC-encoded alldeterminants recognized on the target cells were polymorphic. Evidence that a single MHC haplotype may encode more than one target cell alldeterminant was obtained since two different target specificities were coexpressed in RT1.A cells: one that was shared with RT1.A cells and one that was unique (Fig. 1, B and C). Heterogeneity of NK cells at the recognition level could also be inferred from this study since the different allospecificities were demonstrated in the same NK cell population. However, it was not possible to determine if the different NK recognition specificities were distributed clonally as in the human system or on overlapping subsets of cells. Clarification of this matter may be important for understanding the mechanisms behind the generation of the NK allorecognition repertoire.

There is a close resemblance between the MHC complexes of rats and mice (46, 47). In the rat, class I genes are found in the RT1.A and the RT1.C regions, separated by the RT1.B/D region containing class II and other MHC-associated proteins. In other haplotypes the serologically defined RT1.E and C loci have also been described, and are located to the right of the RT1 complex. The RT1.A cluster is situated to the left of RT1.B/D and encodes the strongly and ubiquitously expressed class I molecules that restrict the reactivity of CD8+ T cells to environmental and minor histocompatibility antigens (48, 49). The large majority of the rat class I genes is situated to the right of the RT1 complex, however, in the same location as the H-2D/L, Qa, Tla group of genes in the mouse (50). Up to now only one example of restriction of T cells (of a minor histocompatibility Ag) by RT1.C has been reported and this has led to the general classification as nonclassical (49). The class I molecules encoded by the RT1.C region have not been defined to the same extent as RT1.A, but several distinct families of class I molecules are expressed. In the RT1.C haplotype, the products of two serologically defined loci are expressed at a low density on the cell surface, <10% the density of RT1.A molecules, but the two loci differ markedly in the degree of polymorphism. The first, named RT1.L, is shared by most haplotypes (43) and thus represents the prototype of an ex-

**Table 4.** LEW.1LM2 Cells Express the NK-defined u Target Specificity

| Effector Strain | RT1 haplotype | Inhibitor Strain | RT1 haplotype | Percent inhibition of lysis of LEW.1U (RT1+) Con A blasts at indicated T/I cell ratio* |
|-----------------|---------------|-----------------|---------------|-----------------------------------------------|
| PVG rnu/rnu     | c             | LEW.1U          | u             | 52 | 71 |
| PVG rnu/rnu     | c             | LEW.1LM2        | lm2           | 51 | 76 |
| PVG rnu/rnu     | c             | LEW             | l             | 13 | 28 |
| PVG rnu/rnu     | c             | PVG             | c             | 0  | 5  |

* The results are median values from three experiments, 65% specific lysis was measured in the absence of inhibitor cells.
pressed oligomophic class I group of antigens in the rat, similar to what has been found for the Qa antigens (51). The gene products of the other locus are remarkably polymorphic, showing an allele-specific pattern of reactivity with antisera and mAbs (44, and Wonigeit, K., unpublished observations). This raises the question as to whether it represents the H-2DL orthologue in the rat (44, 50, 52). It is deleted in the LEW.1LM1 strain (43, 44), and it was of particular interest to us, considering the role of H-2D/L antigens in mouse NK allorecognition models (9, 17, 20). The deletion in the other mutant haplotype, Im2, affects a different portion of the RT1C region, and the polymorphic locus is thus present in the LEW.1LM2 strain (43). It is interesting to note that the NK-defined target specificity was lost in LEW.1LM1 whereas the target specificity was retained in LEW.1LM2. Our results therefore are compatible with the idea that the polymorphic locus to the right of RT1B/D is important in regulating rat NK alloreactivity in vitro.

In certain rat strain combinations, the susceptibility and specificity conferred to the target cells by the RT1C region was to some degree influenced by the left hand side of the RT1 complex, in agreement with findings in mice that the specificity conferred to the target cells by the mouse NK allorecognition models (9, 17, 20). The deletion, however, that interactions between genes in both flanks of the polymorphic region containing among others the I region from the left part of the RT1 complex could reflect the heterogeneity of NK cells in that the reactivity of a minor subpopulation of the cells was controlled by the RT1.A and not the C region in these combinations. It is conceivable, however, that interactions between genes in both flanks of the RT1 complex may take place. We have recently obtained some indications in support of such a possibility. Susceptibility to lysis by alloreactive NK cells mapped to the RT1A region in one combination of rat strains, analogous to the mapping of the H-2K loci to H-2K (56). The NK-defined target specificity expressed in this strain (PVG.R1), disparate from the effector cells (PVG) only in the RT1A region, could not be explained solely by the A region, however. The same target specificity was not expressed in another strain (PVG.R8) with the same A region, identical genetic background, but with different B/D-C regions (Napper, C., Vaage, J. T., unpublished observations).

Different NK recognition models have been developed, reflecting the complexity of the systems studied and possibly also species differences. Solid evidence exists that the expression of certain MHC class I molecules protects against NK lysis in line with the missing self hypothesis, and support for this has recently also been obtained in the rat (57). Two different alternatives are usually considered to explain the findings, namely the delivery of an inhibitory signal by the class I molecule (effector inhibition) or the masking of an NK-activating molecule mediated by the MHC class I antigen (target interference) (4). The target interference model in many respects resembles the Hh recognition model in that the Hh antigens are considered to activate NK lysis (19, 20, 58). Thus, MHC class I genes could be the downregulatory genes referred to within the Hh recognition model (Hh-1r), but the mapping data with intra-H-2 recombinant strains have shown that the Hh-1r loci are different from H-2D (19, 20). We think that the rat model provides more direct evidence that triggering of NK lysis by certain allogeneic MHC alleles does occur. The immunogenetics of ALC in rats differs substantially from that of hybrid and allogeneic resistance in mice. An extensive survey of the F1 hybrid effect in ALC concluded that there is no absolute requirement for homozygous presentation of the ALC determinants on the donor cells for them to be eliminated as in the Hh system in the mouse (40, 59), in line with the present investigation demonstrating recognition of nonrecessively inherited RT1C region-encoded target cell allodeterminants. Furthermore, employing LEW.1LM1 mutant cells, evidence was obtained that the C region genes deleted in the mutant were important in activating the NK cell subset specifically eliminating parental LEW cells. The corresponding NK allolspecificity was missing when we used mutant instead of LEW inhibitor cells (Fig. 6 A). This was also reflected in the reduced susceptibility of mutant cells to direct lysis by the polyyclonal NK cell population used (Table 3). It is important to note, however, that the LEW.1LM1 cells were still susceptible to allogeneic NK cell lysis, suggesting that other NK cell subsets with different recognition specificities were responsible for the "residual" lysis of these mutant cells. The notion that certain polymorphic MHC-encoded alloantigens may provoke rather than inhibit NK lysis is supported by some of the present data, but the biological significance of such a system is not clear. Consistent with this idea, however, the existence of both inhibiting and triggering NK cell receptors has been postulated (60). Furthermore, by drawing an analogy with the T cell alloreactivity phenomenon as a byproduct of antigenic presentation to T cells, it can justifiably be argued that NK cell allorcognition reflects important aspects of NK recognition mechanisms yet to be resolved.

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