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 F_1 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*, *l*, *a*, and *n* haplotypes) could be discerned when we used polyclonal NK cells from the PVG (*RT1^c*) 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 F_1 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 β_2 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,

A preliminary report has been published. (1993. *Transplant. Proc.* 25:2802).

[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.

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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 $H\text{-}2D^d$ 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 Hh-1 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 allodeterminants 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 *rnu/rnu*), 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 F_1 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 veterinary surgeon and registered by the Experimental Animal Board under the Ministry of Agriculture of Norway.

In Vitro Cytotoxicity Assay. The 4-h ^{51}Cr-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 × 10^6 target cells were incubated with ~3.7 MBq ^{51}Cr/ml, washed three times, and 10^4 cells were added to the effector cells (10^5–10^6 cells/well) in U-bottomed 96-well plates. In the cold target inhibition experiments, effector cells (10^6 cells/well) were added first, followed by inhibitor cells before target cells (10^4 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% CO_2/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 × 10^6 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^{-5} 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 Løvik, 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 rIL-2 (38). The number of peritoneal exudate cells (PEC) retrieved, varied between 100 and 1,000 × 10^6 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 NKR-P1 molecule (39), expressed at high density on NK cells. The main contaminants were macrophages staining with OX41 (10–20%) and T cells (20–30%) that stained with mAb against CD3 (1F4 and G4.18), CD5 (Ox19), and TCR-α/β (R73). The percentage of NK cells was higher (70–80%) among PEC from athymic *rnu/rnu* 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% CO_2/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-α/β^-, 3.2.3$^+$ NK cells. In some experiments we used unstimulated nylon wool nonadherent spleen cells from *rnu/rnu* 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 ^{51}Cr/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. ^{51}Cr is retained in viable lymphocytes that resume their recirculation pattern after intravenous injection and 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.

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

Strain	*RT1* haplotype	*RT1* regions			Non-MHC background
		A	*B/D*	*C*	
LEW	*l*	*l*	*l*	*l*	LEW
LEW.1U	*u*	*u*	*u*	*u*	LEW
LEW.1A	*a*	*a*	*a*	*a*	LEW
LEW.1AV1	*av1*	*a*	*a*	*av1*	LEW
LEW.1N	*n*	*n*	*n*	*n*	LEW
LEW.1C	*c*	*c*	*c*	*c*	LEW
LEW.1AR2	*r3*	*a*	*a* ↓*	*u*	LEW
LEW.1WR1	*r4*	*u*	*u* ↓	*a*	LEW
LEW.1WR2	*r6*	*u* ↓	*a*	*a*	LEW
LEW.1R14	*r14*	*u*	*u* ↓	*l*	LEW
LEW.1R15	*r15*	*l*	*l* ↓	*u*	LEW
LEW.1LM1	*lm1*	*l*	*l*	*lm1*	LEW
LEW.1LM2	*lm2*	*l*	*l*	*lm2*	LEW
PVG	*c*	*c*	*c*	*c*	PVG
PVG *rnu/rnu*	*c*	*c*	*c*	*c*	PVG *rnu/rnu*
PVG.1U	*u*	*u*	*u*	*u*	PVG
PVG.1AV1	*av1*	*a*	*a*	*av1*	PVG
PVG.R8	*r8*	*a* ↓	*u*	*u*	PVG
AO	*u*	*u*	*u*	*u*	AO
LOU/C	*u*	*u*	*u*	*u*	LOU/C

* Recombination point

Results

PVG-rnu/rnu NK Cells Discriminate between Four Different RT1-controlled Target Specificities. The ability of a polyclonal NK cell population from PVG *rnu/rnu* (*RT1^c*) rats to discriminate between different MHC congenic target cells, was tested in cold target inhibition experiments. Lysis of the MHC incompatible LEW (*RT1^l*), LEW.1N (*RT1^n*), LEW.1A (*RT1^a*), and LEW.1U (*RT1^u*) 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 (*RT1^c*) 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 *RT1^u* Con A blasts was obtained with unlabeled third party *RT1^n*, *RT1^l*, or *RT1^a* blasts (Fig. 1 *D*) and the specific component in the recognition of *RT1^u* targets (the *u* NK allospecificity) was therefore marked. The *l* NK allospecificity was less pronounced since unlabeled *RT1^a*, *RT1^u*, or *RT1^n* Con A blasts cross-inhibited the lysis of *RT1^l* cells substantially (Fig. 1 *A*). The *n* allospecificity

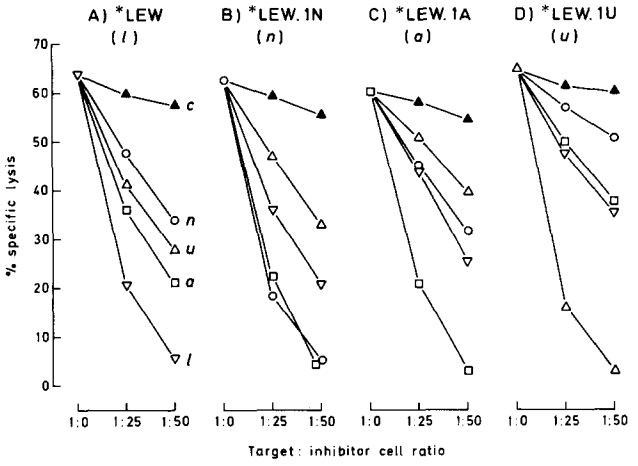


Figure 1. NK cells display four different RT1-controlled allospecificities in cross-competition experiments. As effector cells were used NK cells from the peritoneal cavity of PVG-*rnu/rnu* (*RT1^c*) rats implanted with diffusion chambers containing transfected CHO cells secreting rat rIL-2. Target and inhibitor cells were Con A–activated T cell blasts from MHC congenic rats: LEW (*RT1^l*, ▽), LEW.1N (*RT1^n*, ○), LEW.1A (*RT1^a*, □), LEW.1U (*RT1^u*, △), and LEW.1C (*RT1^c*, ▲). The target cells and their respective *RT1* haplotypes are denoted above each graph. The *RT1* haplotypes of the inhibitor cells are given (*A*). The results are median values from two separate experiments where all the different allospecificities were measured simultaneously.

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 $RT1^n$ cells in inhibiting lysis of $RT1^n$ target cells (Fig. 1 *B*). This was not the case in the opposite direction, since $RT1^n$ 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*-controlled NK allospecificities could be discerned in cross-competition studies, viz., a^+, a^+n^+, l^+, 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 ($RT1^u$) NK cells efficiently lysed $RT1^a$ (LEW.1A), but not $RT1^u$ (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 (*aau*) and PVG.R8 (*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 *uuu* 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; *aau* and *auu* but not *uua* 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 $RT1^c$ 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.

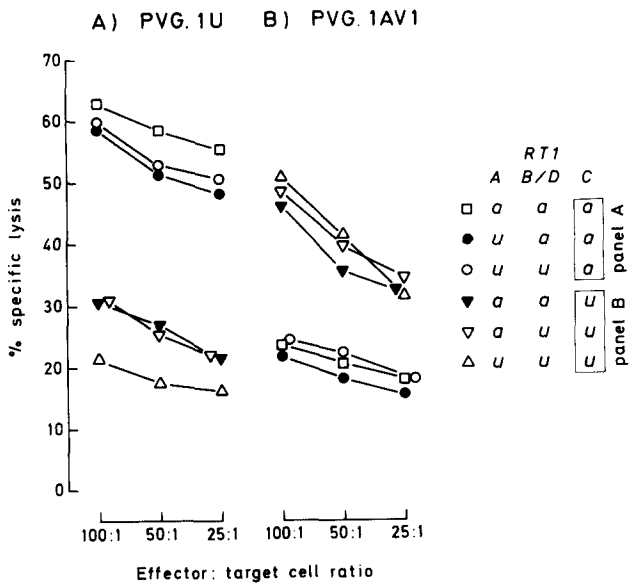


Figure 2. NK susceptibility of lymphoblasts is controlled predominantly by the *RT1.C* region. The effector cells were NK cells from the PVG.1U and PVG.1AV1 strains (denoted above each of the two panels) activated with rat rIL-2 in vitro. Target cells were Con A blasts from different MHC congenic and intra-MHC recombinant strains: LEW.1A (□), LEW.1WR2 (●), LEW.1WR1 (○), LEW.1AR2 (▼), PVG.R8 (▽), and LEW.1U (△). The *RT1* haplotypes of the target cells are given. Representative results from four (*A*) or three (*B*) experiments are shown.

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 (*aau*) lymphoblasts were killed slightly better than fully MHC compatible *uuu* 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 (*uaa*) and LEW.1WR1 (*uua*) Con A blasts were slightly less sensitive to lysis than *aaa* 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 ^{51}Cr and injected intravenously into PVG.1U, AO, and LOU/C rats (all $RT1^u$). 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^u lymphoblasts efficiently inhibited the lysis of $RT1^u$ 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 $RT1^l$-target cells was observed with *uul* and not with *llu* 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 *lll* cells whereas *llu* lymphoblasts inhibited slightly better than *uuu* 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		Donor					LN index		
	RT1		*RT1*	*RT1* regions			Cervical	Mesenteric	
Strain	haplotype	Strain	haplotype	*A*	*B/D*	*C*	LN	LN	No.*
PVG.1U	*u*	LEW.1A	*a*	*a*	*a*	*a*	0.093	0.14	2
PVG.1U	*u*	LEW.1WR2	*r6*	*u*	*a*	*a*	0.17	0.19	2
PVG.1U	*u*	LEW.1WR1	*r4*	*u*	*u*	*a*	0.14	0.16	2
PVG.1U	*u*	LEW.1AR2	*r3*	*a*	*a*	*u*	0.54	0.64	2
PVG.1U	*u*	PVG.R8	*r8*	*a*	*u*	*u*	0.91	0.88	2
AO	*u*	LEW.1WR2	*r6*	*u*	*a*	*a*	0.086	0.090	1
AO	*u*	LEW.1WR1	*r4*	*u*	*u*	*a*	0.11	0.094	2
AO	*u*	LEW.1AR2	*r3*	*a*	*a*	*u*	0.37	0.37	1
AO	*u*	PVG.R8	*r8*	*a*	*u*	*u*	0.77	0.72	1
AO	*u*	LEW.1U	*u*	*u*	*u*	*u*	0.66	0.61	1
LOU/C	*u*	LEW.1WR2	*r6*	*u*	*a*	*a*	0.10	0.11	2
LOU/C	*u*	LEW.1WR1	*r4*	*u*	*u*	*a*	0.18	0.18	2
LOU/C	*u*	LEW.1AR2	*r3*	*a*	*a*	*u*	0.63	0.65	2
LOU/C	*u*	PVG.R8	*r8*	*a*	*u*	*u*	1.2	1.2	1
LOU/C	*u*	LEW.1U	*u*	*u*	*u*	*u*	1.3	1.2	2

ALC was calculated as the uptake of intravenously injected ^{51}Cr-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.

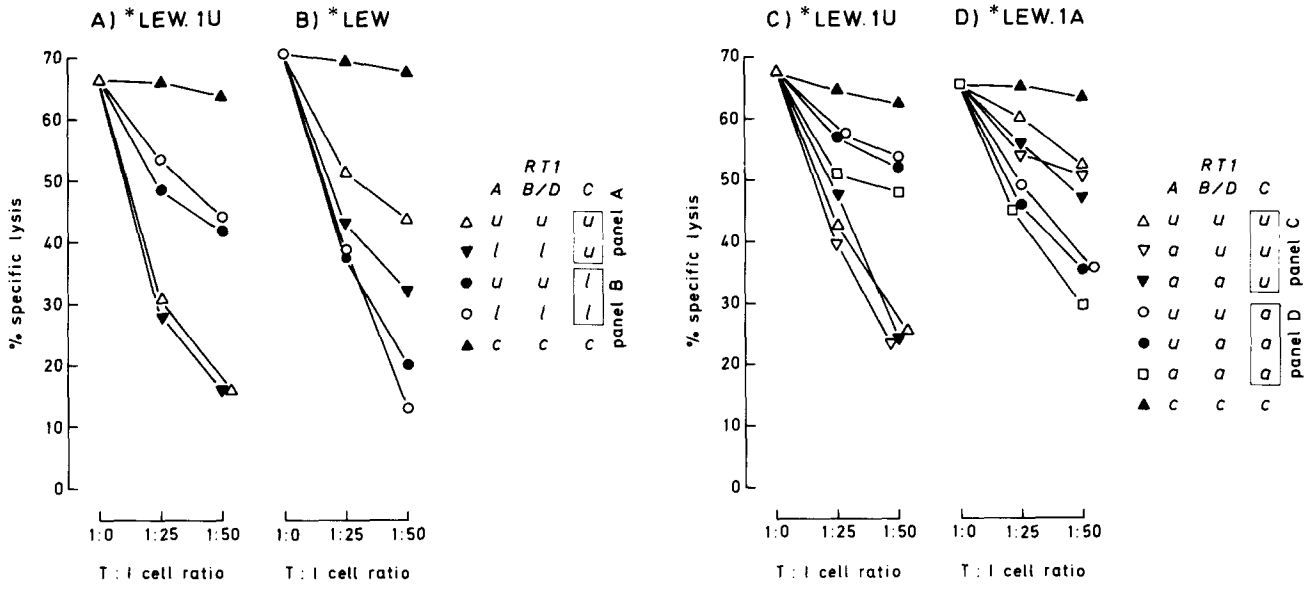
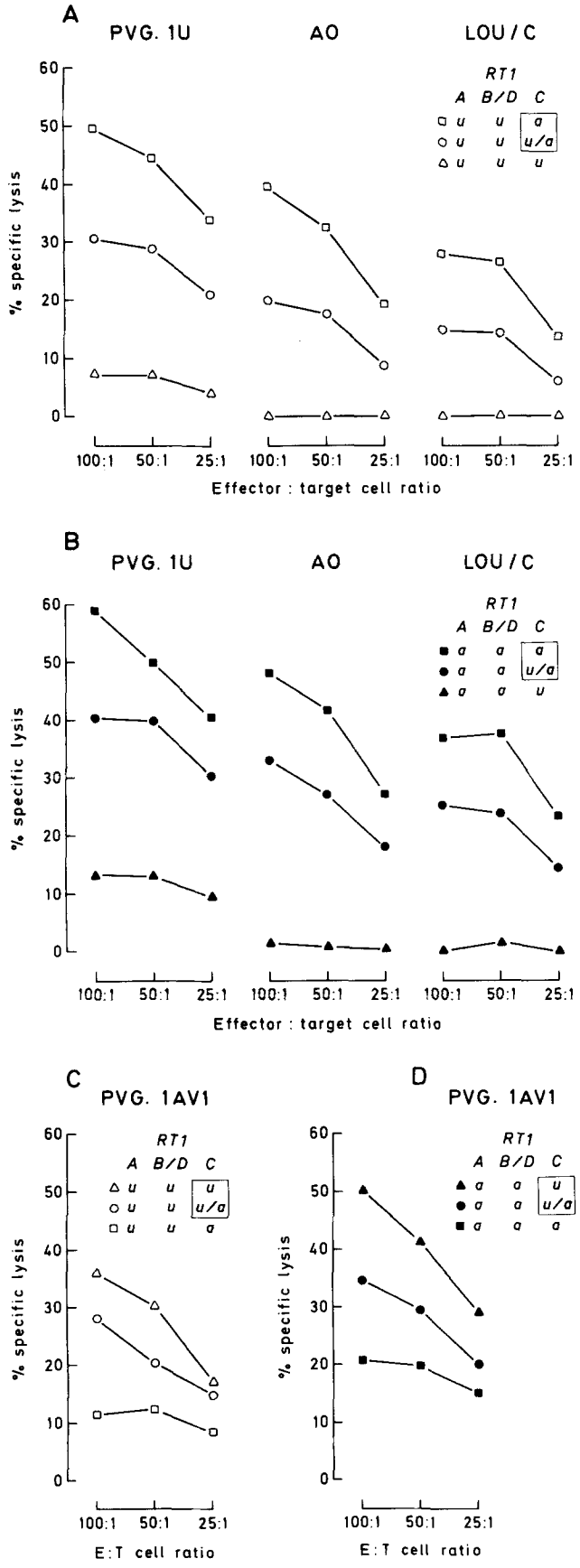


Figure 3. NK-defined target cell specificities are controlled predominantly by the *RT1.C* region. Effector cells were as in Fig. 1. Target and inhibitor cells were Con A blasts from two different panels of intra-MHC recombinant strains and from the respective parental strains: (*A* and *B*) LEW.1U (△), LEW.1R15 (▼), LEW.1R14 (●), LEW (○), and LEW.1C (▲). (*C* and *D*) LEW.1U (△), PVG.R8 (▽), 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).



ments controlling susceptibility to direct lysis by PVG.1U NK cells (Fig. 2 *A*).

Nonrecessive Inheritance of RT1.C Region-controlled Target Cell Susceptibility and NK-defined Target Specificities. NK cells were generated from $RT1^u$ (PVG.1U, AO, and LOU/C), $RT1^{av1}$ (PVG.1AV1) and $RT1^c$ (PVG *rnu/rnu*) strains. Target and inhibitor cells were from two different crosses designed to generate F_1 hybrids with heterozygosity only in the *RT1.C* region, i.e., the (LEW.1U × LEW.1WR1)F_1 (*uuu/a*) and (LEW.1AR2 × LEW.1A)F_1 (*aau/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 (*uua*) Con A blasts, but not *C* region-compatible LEW.1AR2 (*aau*) and LEW.1U (*uuu*) lymphoblasts (Fig. 4, *A* and *B*). Lymphoblasts from *RT1.C* region heterozygous F_1 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 $RT1^{av1}$ NK cells recognizing allodeterminant(s) coded for by C^u genes. PVG.1AV1 NK cells lysed the two different F_1 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 (*aau*) and LEW.1A (*aaa*) strains expressed different NK-defined target specificities as detected by their relative inability to effectively cross-compete for the $RT1^c$ effector cells in cold target inhibition experiments (Fig. 5, *A* and *B*). The (LEW.1AR2 × LEW.1A)F_1 hybrid (*aau/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 F_1 cells was measured, as expected, since the F_1 cells expressed both parental target specificities (Fig. 5 *C*).

Loss of an NK-defined Target Specificity in an RT1.C Region Class I-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 ($RT1^{lm1}$) strain, derived from the LEW strain, is characterized by a genomic deletion of 100 kb of class I crosshybridizing 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 × LEW.1WR1)F_1 (○), and LEW.1U (△). (*B* and *D*) LEW.1A (■), (LEW.1AR2 × LEW.1A)F_1 (●), and LEW.1AR2 (▲). Representative experiments are shown.

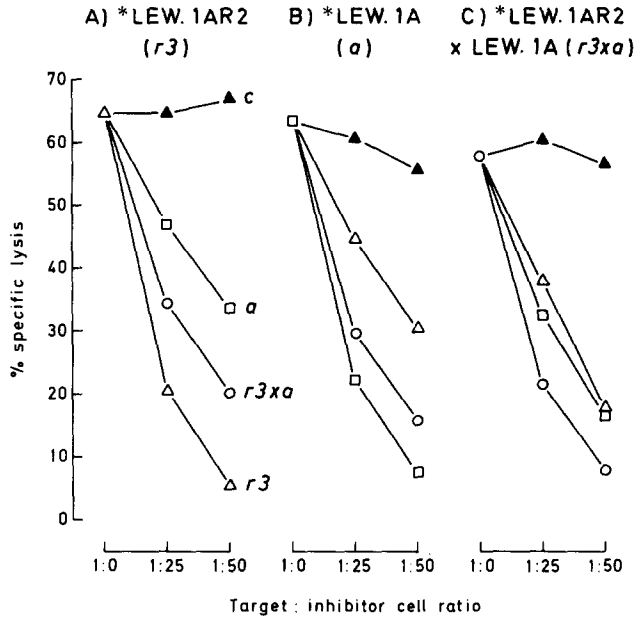


Figure 5. Demonstration of nonrecessive inheritance of parental NK-defined target specificities controlled by the *RT1.C* region. Effector cells were as in Fig. 1. Con A blasts were used as target and inhibitor cells: LEW.1AR2 (△), LEW.1A (□), (LEW1.AR2 × LEW.1A)F_1 (○), and PVG (▲). Labeled target cells 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 *l* 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 *l* target specificity rather selectively.

The LEW.1LM2 ($RT1^{lm2}$) mutant strain is derived from the LEW.1LV3 ($RT1^{lv3}$) and LEW.1U ($RT1^{u}$) 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 *RT1.A-B/D* regions of LEW.1LM2 are derived from LEW.1LV3 whereas most of the *RT1.C* 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 $RT1^{u}$ targets as efficiently as did $RT1^{u}$ 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

Table 3. *Lower NK Sensitivity of LEW.1LM1 than LEW Lymphoblasts to Alloreative NK Cells*

Effector		Target		Percent specific lysis at indicated E/T cell ratio*	
Strain	*RT1* haplotype	Strain	*RT1* haplotype	50:1	25:1
PVG *rnu/rnu*	*c*	LEW	*l*	60 (53–78)	46 (18–62)
PVG *rnu/rnu*	*c*	LEW.1LM1	*lm1*‡	55 (41–68)	35 (20–50)
PVG *rnu/rnu*	*c*	LEW.1U	*u*	52 (46–67)	38 (19–57)
PVG *rnu/rnu*	*c*	LEW.1C	*c*	8 (3–29)	4 (0–17)
AO	*u*	LEW	*l*	48	28
AO	*u*	LEW.1LM1	*lm1*‡	18	1
AO	*u*	LEW.1U	*u*	8	4
AO	*u*	LEW.1C	*c*	52	27

Con A–activated T cell blasts were used as target cells. Effector cells were NK cells from the peritoneal cavity of PVG *rnu/rnu* or AO rats implanted with diffusion chambers containing rat rIL-2–secreting CHO cells.

* The results are medians and ranges (given in parentheses) from four (PVG *rnu/rnu*) or one experiment(s) (AO).

‡ The percent specific lysis of LEW.1LM1 was statistically lower than the lysis of LEW lymphoblasts (combined data from PVG *rnu/rnu* and AO: E/T 50:1, $p < 0.001$; E/T 25:1, $p < 0.01$).

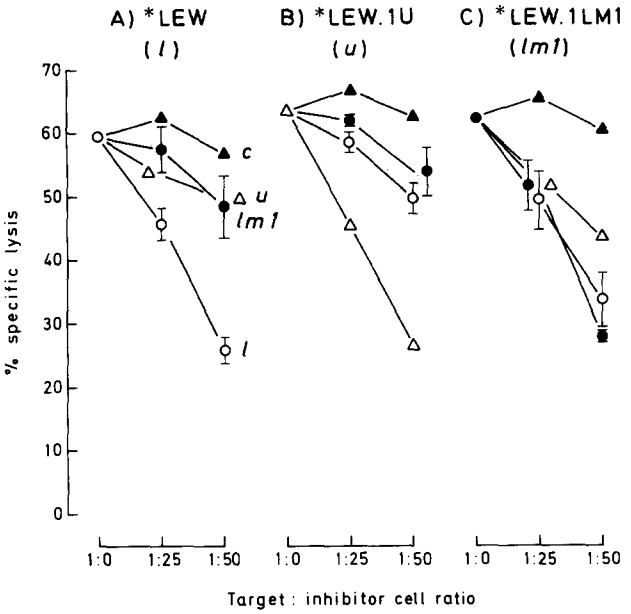


Figure 6. Loss of an NK-defined target specificity in the *RT1.C* region deletion mutant LEW.1LM1. Effector cells were as in Fig. 1. Con A blasts were used as target and inhibitor cells: LEW (○), LEW.1U (△), LEW.1LM1 (●), and LEW.1C (▲). Labeled target cells are denoted above each graph. The results are medians from two experiments, but the results in *A* and *B* were confirmed in three additional experiments. To facilitate the comparison between LEW and LEW.1LM1, ranges are indicated.

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 allodeterminants recognized on the target cells were polymorphic. Evidence that a single MHC haplotype may encode more than one target cell allodeterminant was obtained since two different target specificities were coexpressed in $RT1^a$ cells: one that was shared with $RT1^n$ 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 *G* 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 $RT1^l$-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		Inhibitor		Percent inhibition of lysis of LEW.1U ($RT1^u$) Con A blasts at indicated T/I cell ratio*	
Strain	*RT1* haplotype	Strain	*RT1* haplotype	1:25	1:50
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 oligomorphic 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-2D,L* 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 *RT1.C* region, and the polymorphic locus is thus present in the LEW.1LM2 strain (43). It is interesting to note that the NK-defined *l* target specificity was lost in LEW.1LM1 whereas the *u* target specificity was retained in LEW.1LM2. Our results therefore are compatible with the idea that the polymorphic locus to the right of *RT1.B/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 *RT1.C* region was to some degree influenced by the left hand side of the *RT1* complex, in agreement with findings in mice that the strength of resistance to *Hh-1/H-2D*–incompatible BMC could be influenced by the left part of the *H-2* (53). In the *a–u* recombinants, the additional effect was due to the classical class I region (*RT1.A*) (Figs. 2 *A* and 3 *D*), and not the *B/D* region containing among others the *cim* locus, representing a polymorphism in the *Tap 2/mtp2* transporter gene and affecting the spectrum of peptides bound to the RT1.A^a molecule (54, 55). Thus, in the one informative set of recombinants, the different *cim* alleles in the $RT1^a$ and $RT1^u$ haplotypes did not affect the NK-defined target phenotype. The influence 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 *RT1.A* region in one combination of rat strains, analogous to the mapping of the *Hh-3* locus to *H-2K* (56). The NK-defined target specificity expressed in this strain (PVG.R1), disparate from the effector cells (PVG) only in the *RT1.A* 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 (Naper, 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 F_1 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 *RT1.C* 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 *l* NK allospecificity 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 polyclonal 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 allorecognition reflects important aspects of NK recognition mechanisms yet to be resolved.

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