An Autosomal Dominant Locus, *Nka*, Mapping to the Ly-49 Region of a Rat Natural Killer (NK) Gene Complex, Controls NK Cell Lysis of Allogeneic Lymphocytes

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Summary

Natural Killer (NK) cells can recognize and kill MHC-incompatible normal bone marrow-derived cells. Presently characterized MHC-binding receptors on NK cells, including the Ly-49 family in the mouse, transmit inhibitory signals upon binding to cognate class I MHC ligands. Here we study in vivo NK-mediated lysis of normal allogeneic lymphocytes in crosses between alloreactivity-competent PVG rats and alloreactivity-deficient DA rats. NK cells from both strains are able to lyse standard tumor targets. We identify an autosomal dominant locus, *Nka*, that controls NK-mediated alloreactivity. Individuals carrying the dominant PVG allele in single dose were fully competent in eliminating allogeneic target cells, suggesting that *Nka* encodes or regulates a gene product inducing or activating alloreactivity. By linkage analysis and pulsed field gel electrophoresis, a natural killer gene complex (NKC) on rat chromosome 4 is described that contains the rat NKR-P1 and Ly-49 multigene families plus a rat NKG2D homologue. *Nka* maps within the NKC, together with the most telomeric Ly-49 family members, but separate from NKG2D and the NKR-P1 family. The *Nka*-encoded response, moreover, correlates with the expression of transcripts for Ly-49 receptors in NK cell populations, as Northern blot analysis demonstrated low expression of Ly-49 genes in DA NK cells, in contrast to high expression in alloreactivity-competent PVG, (DA × PVG)F_1, and PVG.1AV1 NK cells. The low Ly-49 expression in DA is not induced by MHC haplotype, as demonstrated by high expression of Ly-49 in the DA MHC-congenic PVG.1AV1 strain. Finally, we have cloned and characterized the first four members of the rat Ly-49 gene family. Their cytoplasmic domains demonstrate substantial heterogeneity, consistent with the hypothesis that different Ly-49 family members may subserve different signaling functions.

Natural killer (NK)[1] cells are a subpopulation of lymphocytes that without prior sensitization lyse various target cells (1), including mature allogeneic hematopoietic cells (2–4). NK alloreactivity, here defined as the lysis of allogeneic cells, is directed against class I major histocompatibility complex (MHC) gene products on the target cells, and displays immunological specificity, with clonally distributed target cell specificities (4–7). Despite the many properties in common with T cell alloreactivity, it does not involve the αβ or γδ T cell receptors (3, 8, 9). Another feature distinguishing NK from T cell alloreactivity is that the MHC-binding receptors on NK cells as a rule seem to transmit inhibitory signals upon ligation to their cognate class I MHC ligands. Thus, expression of self class I MHC antigens on target cells generally inhibits lysis by NK cells, conforming with the "missing self" hypothesis on NK recognition (10). Because the target specificities on allogeneic cells are non-self MHC molecules, NK alloreactivity can also be accommodated into this hypothesis.

There is experimental evidence that at least three types of NK cell receptors for class I MHC can mediate this inhibition: Killer-cell inhibitory receptors (KIR) on human NK cells (11, 12), and mouse Ly-49A (mLy-49A) (13). The Ly-49 multigene family belongs to a group of membrane proteins related by their structure (type II integral membrane proteins with an external C-type lectin domain) and genetic proximity, as well as by their expression on NK cells, collectively referred to as NK lectin-like receptors (NKLLR). The region encoding this receptor super-

[1] *Abbreviations used in this paper:* CRD, carbohydrate recognition domain; KIR, killer-cell inhibitory receptors; NK, natural killer; NKC, natural killer gene complex; NKLLR, NK lectin-like receptors; PFGE, pulsed field gel electrophoresis; TDL, thoracic duct lymphocytes.

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family has been termed the natural killer gene complex (NKC) (14). The mouse NKC includes the mNKR-P1 (14, 15) and mLy-49 (16, 17) gene families as well as CD69 (18), and resides on chromosome 6 (14, 18). In the human, the NKG2 family (19), CD94 (20), NKR-P1A (21), and CD69 (22) map to an NKC on chromosome 12. In particular, the Ly-49 family has been promoted as specific receptors responsible for NK lysis of allogeneic cells in the mouse, based on cytotoxicity studies (13, 23) and the demonstrations that the Ly-49 loci are highly polymorphic (24) and that Ly-49 molecules specifically bind certain allelic variants of class I MHC molecules (17, 25, 26). In addition, the level of expression of individual Ly-49 family members on NK cells is influenced by MHC haplotype (27–29), suggesting a shaped repertoire. In contrast to Ly-49 in the mouse, MHC-binding inhibitory NK receptors in the human, the recently cloned KIR molecules, are members of the Ig superfamily (30–32), suggesting that the same function is mediated by different types of molecules in the two species, or, alternatively, a redundancy of MHC-binding receptors.

NK allorecognition is further complicated by evidence indicating that the expression of class I MHC molecules on target cells can also promote, rather than inhibit, their lysis by NK cells. In the rat, a gene (or genes) within the MHC renders lymphocytic blast targets susceptible to lysis by allogeneic NK cells (6). Whereas the roles of mLy-49A and the human KIR family have mainly been studied in vitro, we have here studied rat NK alloreactivity in vivo. We have cloned the first four rat Ly-49 genes, identified and mapped a rat NKC, and present evidence that products encoded in the Ly-49 subregion of the NKC may activate NK-mediated alloreactivity in the rat.

Materials and Methods

Animals. The inbred rat strains PVG, PVG.1AV1, DA, AO, and LEW, and (DA × PVG)F_1 and F_2 hybrids were reared under conventional conditions (routinely screened for pathogens) in Oslo or obtained commercially (Harlan Olac, Bicester, UK). (DA × PVG)F_1 × DA rats were generated from breeding pairs of male (DA × PVG)F_1 and female DA rats.

In Vivo NK Alloreactivity Assay. Donor thoracic duct lymphocytes (TDL) were freshly isolated (33), labeled with ^{51}Cr (34) or ^{111}In, and then injected as 1.5×10^7 cells in 1 ml of PBS into the lateral tail veins of 6–10-wk-old recipient rats. By 24 h the recipients were killed by CO_2 overdosage and the right kidney and superficial cervical lymph nodes were removed. The organs were weighed and the radioactivity in each sample determined. ^{111}In-labeling was for 10 min at room temperature, with 1×10^8 cells and 5 μCi of ^{111}In-Oxine (Amersham International, Little Chalfont, UK) per ml of PBS, followed by three washes in PBS supplemented with 5% FCS. Simultaneous counting of both ^{51}Cr and ^{111}In was performed by setting the counting windows at 285–370 keV and 390–480 keV, respectively. Spillover from ^{111}In into the ^{51}Cr window was subtracted based on emission profiles (Cobra Auto-Gamma counter, Packard Instruments, Downers Grove, IL).

LAK Cells. IL-2-activated cells were cultured from nylon wool-passed spleen cells negatively selected for T cells by immunomagnetic beads (Dynal, Oslo, Norway) after incubation with mAb towards the αβ TCR (R73 [35]) and CD5 (OX19 [36]), yielding a >99% $CD3^-$ population (flow cytometry using mAb 1F4 (37); FACScan, Becton Dickinson, Mountain View, CA). Cells were cultured at 37°C in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS (GIBCO BRL), 1 mM Na pyruvate, 2 mM glutamine, 5×10^{-5}M 2-ME, and rat rIL-2 (38) at concentrations equivalent to 1,000 IU/ml of human rIL-2. At day 14, cells were harvested, and bright expression of NKR-P1 in >98% of the cells was verified by flow cytometry using the mAb 3.2.3 (39).

cDNA Cloning and Sequence Analysis. The nucleotide sequences of rLy-49 genes were derived by homology screening of an F344 rat NK cell cDNA library and by specific priming of the PCR as previously described (14). IL-2-activated NK cells from F344 rats were generated as described (40), and polyadenylated mRNA was produced using the Fast Track mRNA isolation method (Invitrogen, San Diego, CA). An NK cell cDNA library (complexity 1.3×10^6) was produced using a λZAP cDNA Library Kit (Stratagene, La Jolla, CA) according to the manufacturers' instructions. 2.5×10^5 plaques were screened with a radiolabeled XhoI/HindIII fragment (nucleotides 66–1137) of the mLy-49A cDNA (41), with the final wash in 0.25 × SSC, 0.1% SDS at 45°C, as described (14). Three clones, rLy-49.9, rLy-49.12a, and rLy-49.19 were purified to homogeneity. Both strands of each cDNA were sequenced using the Sequenase 2.0 system (United States Biochemical, Cleveland, OH), and sequences were analyzed using the Socrates Datalink facilities at the UCSF Computer Center (San Francisco, CA). The nucleotides encoding the cytoplasmic domain of rLy-49.12a were frame-shifted compared to those encoding the homologous stretch of mouse Ly-49A. To rule out a cloning artifact, the sequence specific oligonucleotides 5′-ATCAGGGAAGACCACCAGCTGTCCCTCAATTGG-3′ and 5′-TTTGTTATGGTTCTTTATTTAGTTCTGTGG-GAGTTTATGC-3′ were used to selectively amplify additional variants of rLy-49.12 from F344 rat NK cell cDNA according to standard methods. The library-derived rLy-49.12a cDNA and the PCR-derived rLy-49.12b cDNA were exactly identical, except that these clones contained non-overlapping internal deletions. The consensus sequence presented here is the predicted, nondeleted rLy-49.12 cDNA deduced from the sequences of rLy-49.12a and rLy-49.12b. When compared to the rLy-49.12 consensus sequence, the library-derived rLy-49.12a contained two internal deletions spanning nucleotides 250–260 and nucleotides 331–493. Each of these deletions shifts the open reading frame of the predicted rLy-49.12 protein. The PCR-derived rLy-49.12b does not contain the upstream deletions (250–260 and 331–493) encoded in rLy-49.12a, but contains a nonoverlapping deletion spanning nucleotides 801–954 of the consensus sequence. The rLy-49.19 cDNA sequence predicts a truncated protein. Additional rLy-49.19 clones were generated by PCR using the rLy-49.19-specific oligonucleotides 5′-AGAAGATACTCCAAATACTCC-CAAGATGAATG-3′ and 5′-TGTCTTGTCTCCAGAGGG-AAGGAAGATGTCC-3′. Although no rLy-49.19 clones other than the rLy-49.19 pseudogene were obtained, one novel cDNA, termed rLy-49.29, was identified among the PCR clones.

Southern Blot Analysis. DNA was extracted from rat liver, digested with restriction endonuclease (New England Biolabs, Beverly, MA), subjected to horizontal agarose gel electrophoresis, and then blotted onto nylon membranes (Biotrans membranes; ICN Biomedicals, Irvine, CA) by conventional methods as previously detailed (9). A Southern blot containing EcoRI-digested genomic DNA from several species was obtained commercially

(Clontech Laboratories, Palo Alto, CA). Hybridization to radiolabeled probe was performed in 50% formamide, 5× SSC, 50 mM sodium phosphate pH 6.5, 250 μg/ml sonicated salmon testes DNA (Sigma Chemical Co., St. Louis, MO), 5× Denhardt's solution, and 0.1% SDS at 42°C for 16–20 h in a hybridization oven. Membranes were washed 4 × 5 min in 2× SSC, 0.1% SDS at room temperature, then 2 × 30 min at 50°C in 0.1× SSC, 0.1% SDS for rat probes, and, typically, 0.5–0.8× SSC, 0.1% SDS for mouse or human probes. Where low stringency hybridization was required, hybridization temperature was lowered to 37°C, and final washes were carried out at 45°C in 1× SSC, 0.1% SDS for 2 × 30 min.

Northern Blot Analysis. Extraction of total cellular RNA, formaldehyde agarose gel electrophoresis and transfer to nylon membrane was performed by conventional methods (9), and hybridization to radiolabeled probe and washing was performed as for Southern blots. Probe removal before rehybridization was for 1 h at 65°C in 50% formamide, 10 mM sodium phosphate, pH 6.5. Filters were hybridized to a chicken β-actin probe (42) to assure equal levels of RNA. Films were preflashed to $A_{540} = 0.15$ (Amersham).

Pulsed Field Gel Electrophoresis (PFGE). PFGE was performed by standard methods (43), using single-cell suspensions from BN, PVG, or DA rat lymph nodes embedded in agarose plugs. Horizontal agarose gels were run in a Rotaphor R22 electrophoresis chamber (Biometra, Göttingen, Germany), with microprocessor-controlled cooling system, ramping of voltage, switch time, and field angle. DNA was transferred to nylon membrane by exposing the gel to 302 nm UV light for 45 s, denaturing in 0.5 M NaOH, 1.5 M NaCl, neutralizing in 3 M sodium acetate, pH 5.5, followed by capillary transfer in 20× SSC buffer for 24 h, and hybridized as for ordinary Southern blots.

Probes. The following probes were radiolabeled with (α-^{32}P)-dCTP by a random nonamer primer protocol (Megaprime DNA Labelling System; Amersham): P1B, an NKR-P1B-specific 287-bp 3′UTR fragment of a rat NKR-P1B (rNKR-P1B) cDNA (J.C. Ryan and W.E. Seaman, unpublished observation; GenBank accession number: U56936); rLy-49.9 and rLy-49.12 (library-derived) cDNAs; 49-CTM, a fragment containing the cytoplasmic and transmembrane regions (nucleotides 1–376) of rLy-49.9; 5E6-5′, a 142-bp BsmAI fragment from the 5′UTR of a mLy-49C cDNA (44); human NKG2D (hNKG2D), hNKG2A, and hNKG2C cDNAs (45); a rat Ig κ C-region fragment (46); and rat KAP (kidney androgen-regulated protein; reference 47), CD9 (48), PTHLH (parathyroid hormone-like peptide; reference 49), and NKR-P1A (50) cDNAs. The 5E6-5′ probe yielded bands that did not hybridize to the rLy-49.9, -12 or -19 cDNA probes, and identified a subgroup of Ly-49 genes.

Statistics and Linkage Map Calculation. The probability of nonlinkage was calculated by the χ^2 test. Map distances in centiMorgans (cM) were calculated from recombination frequencies using the Kosambi linkage function (51).

Results

NK-mediated Lysis of Allogeneic Cells Is Controlled by a Dominant Locus, Nka. In the rat, natural killing of resting allogeneic lymphocytes can be assessed in vivo (52) as well as in vitro (34). Combinations of effector and target cells from different inbred strains elicit different responses, indicating that the response is under genetic control. A difference in MHC haplotype between effector and target strains is necessary, but not always sufficient, for lysis. As demonstrated both in vivo and in vitro, PVG NK cells efficiently lyse lymphocytes from several MHC-incompatible donor strains, including AO and LEW, whereas DA NK cells do not (6, 34, 52, 53).

To further define the control of this response, we assessed the in vivo NK cell alloreactivity by offspring of PVG and DA rats against AO thoracic duct lymphocytes (TDL). All (DA × PVG)F_1 rats displayed high levels of alloreactivity, equal to that of parental PVG rats. Of 53 F_2 rats, 10 showed low and 43 high alloreactivity (Fig. 1 *A*). Of 201 (DA × PVG)F_1 × DA back-crossed rats, 96 showed high reactivity (Fig. 1 *B*). These results are close to 1:3 and 1:1 distributions, respectively, and demonstrate that high alloreactivity segregates as a single autosomal dominant locus, with the PVG allele encoding a high reactivity phenotype. We have called this locus *Nka*, for natural killer alloreactivity.

PVG NK cells lyse LEW as well as AO lymphocytes, and discriminate between the two allotypes (2, 6), implying that different recognition molecules are involved. To determine if the responses to these allotypes segregated independently, 19 (DA × PVG)F_1 × DA recipients receiving ^{51}Cr-labeled AO cells were simultaneously injected with ^{111}In-labeled LEW lymphocytes, and retention of the two isotopes in the recipient tissues was determined separately. In addition, 9 rats were injected with LEW cells alone. Of the 28 rats, 12 showed high and 16 low reactivity against LEW cells, close to the expected 1:1 distribution for a single locus showing simple dominant/recessive inheritance. Among the 19 rats that received both AO and LEW cells, there was no segregation of the responses. All 14 rats with low reactivity against AO cells also demonstrated low reactivity against LEW cells, whereas the remaining 5 rats had high reactivity against both (not shown). Thus, alloreactivity to AO and LEW cells is controlled by a single gene or by closely linked genes.

Molecular Cloning and Comparison of Rat Ly-49 cDNAs. We hypothesized that *Nka* might be encoded within the NKC. To prepare for mapping of the rat NKC, we isolated four unique rat homologues of the murine NK receptor Ly-49A (41). The rLy-49.12 sequence is a consensus sequence derived from the library-derived cDNA rLy-49.12a and the PCR clone rLy-49.12b, identical to each other apart from nonoverlapping internal deletions (see Materials and Methods). The nucleotide sequences of the rat Ly-49 cDNAs (rLy-49.9, rLy-49.12, rLy-49.19, and rLy-49.29) were 68–94% identical to each other and to that of mLy-49A (Fig. 2 *B*). As with members of the mouse Ly-49 family, the rLy-49.9, rLy-49.12, and rLy-49.29 cDNAs encode structurally similar polypeptides with putative extracellular calcium-dependent (C-type) lectin domains. rLy-49.19, which is most closely related to rLy-49.12, is a pseudogene in that a stop codon truncates the translated peptide at amino acid 31 (not shown). The predicted mLy-49A, rLy-49.9, -12, and -29 proteins are 62–81% identical. The rat Ly-49-proteins diverge in both the cytoplasmic and lectin domains (Fig. 2, *A* and *C*). Whereas the extracellular lectin

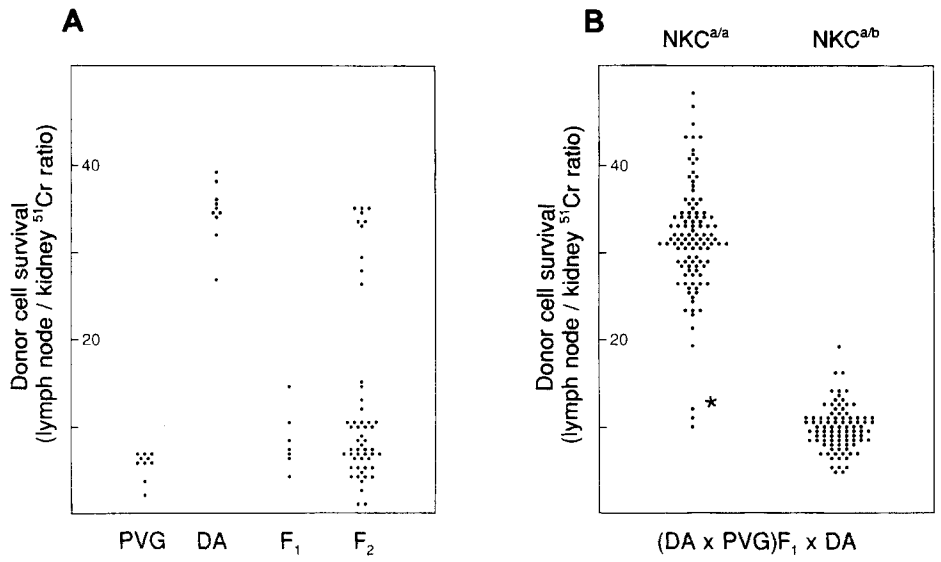

Figure 1. In vivo NK alloreactivity assay towards AO donor TDL. (*A*) Alloreactivity levels in PVG (n = 10), DA (n = 10), (DA × PVG)F_1 (n = 7), or (DA × PVG)F_2 (n = 53) recipients. (*B*) (DA × PVG)F_1 × DA rats (n = 201) were injected with AO donor TDL. The animals were later genotyped (see below) as either heterozygous at the natural killer gene complex (NKC) (denoted $NKC^{a/b}$, n = 93) or homozygous for the DA NKC haplotype ($NKC^{a/a}$, n = 108). Alloreactivity was expressed as the cervical lymph node/kidney (cpm/mg tissue) ratio. High values reflect survival of the donor lymphocytes (homing to lymph nodes), low values reflect rejection and secretion of released ^{51}Cr through the kidneys (52). The results have been normalized by multiplying data from each individual experiment by a linear factor ($40/[m_{high} + m_{low}]$) calculated from the median values of the high and low alloreactivity groups. Genotyping was by Southern blot RFLP analysis (Fig. 5). The three observations marked with an asterisk probably represent false positive values (see Fig. 5).

A

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rLy-49.9      1  MSEQEVTYSSVRFSKSSGLQNQVKPEETKGPKEAGHR ECYVPWHLIVIALGILCTLLLLTVAVLVTI IFQYSQEKHELQETL*NHHHNCSAMQRDIDLKE
rLy-49.12     1  -N--RF-F-TA--H---V----ERT---QR-RK--N- V-***-QIT-T------FFR-VS---M-IN ---------------S-L---Y-T--N--N---
rLy-49.29     1  -N----IF-TE--H---------R----QSSRK--P- V-S--CL--------**P-R-VIA-A--SH ------K--G---IP*---------S--S------
mLy-49A       1  ---------M---H--A---K--R--------R---Y- R-SFH-KF-------F-F---VA-S--AIK ----D-Q-*K---F-*---N---N--S--N--D
                 exon 2                                 exon 3                          exon 4

rLy-49.9    100  EMLRKISIDCSPGNDLLESFNRSKNRWYSKTKAVVNSSQHK GSEIETYWFCYGIKCYYVIKDGKSWDECKQTCQNSSLFLLKIDDEDE RKFLQQQLIPDN
rLy-49.12    98  ----DM-TEY-AV-HF-DFL--E---C-N---T-LD----S -RGV-MH----------F-M-R-T-SG-T-----F---P--T------ LM--HLLVT--S
rLy-49.29    98  -----K-----T---F---LK-EQK-------T-----P-T ----------------------------------------------------- ---------T--
mLy-49A      99  ---KNK-ME-*****-----L--DQ--L-N---T-LD-L--T -RGDKV------M----FVM-R-T-SG------S---S--------- L----LVVPS-S
                                                 exon 5                                                  exon 6

rLy-49.9    200  YWIGFSYDKEKKEWAWIENGPSK LASNTMKFNKKLGGCVFLSKTRLDHTDCINLYSCICGKKLNKFPDLPLQLVLK+    275
rLy-49.12   198  ----L---NK-SD-T--D-N--- --L--R-Y-I-D----------NIN-D--F------R-D----+            265
rLy-49.29   198  --------------------HLN -P-------E-------------------------------------LSN+       269
mLy-49A     195  C-V-L---NK--D----D-R--- --L--R-Y-IRD---ML-------NGN-DQVFI-----R-D---H+           262
                                         exon 7
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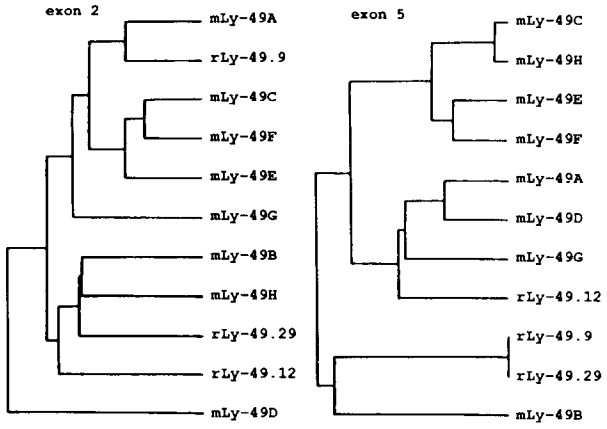
B

cDNA	rLy-49.9	rLy-49.12	rLy-49.19	rLy-49.29
rLy-49.9	100/100			
rLy-49.12	71/63	100/100		
rLy-49.19	68/--	94/--	100/--	
rLy-49.29	90/81	70/62	71/--	100/100
mLy-49A	71/69	69/71	68/--	74/60
mLy-49B	69/53	65/51	64/--	69/46
mLy-49C	72/62	69/60	69/--	73/58
mLy-49D	73/61	78/69	77/--	76/61
mLy-49E	73/64	73/62	69/--	75/60
mLy-49F	74/59	72/60	70/--	75/59
mLy-49G	79/60	78/65	76/--	78/58
mLy-49H	70/60	66/56	66/--	71/60

C



domain of rLy-49.9 is nearly identical to that of rLy-49.29 at the amino acid (96%) and at the nucleotide (98%) level, the Ly-49.9 cytoplasmic domain is more similar to the corresponding domains of mLy-49A, -C, -E, -F, and -G than to those of rLy-49.12 or -29. The cytoplasmic domains of rLy-49.9, mLy-49A, and mLy-49D, but not of rLy-49.12 or rLy-49.29, encode putative inhibitory G-protein (G_i) binding motifs (Fig. 2 *A*). This 10–26–amino acid motif is defined by at least two individual basic residues upstream of BBXB, BBXXB, or BBXXZ, where B is a basic amino acid, X is non-basic, and Z is an aromatic amino acid (54, 55). The structural requirements for this motif are contained in amino acids 29–40 (**KGPKEAGHRECY**) of rLy-49.9 and in amino acids 29–41 (**KGPREAGYRRCSF**) of mLy-49A. Although the functional significance of this motif is not known, the divergence of the rLy-49 cytoplasmic domains suggests that distinct Ly-49 receptors may deliver different signals to NK cells.

A Rat Gene Crosshybridizes to Human NKG2D. Southern blots of genomic DNA from monkey, rat, mouse, dog, cow, and rabbit produced one or more bands hybridizing to hNKG2D. No hybridization was detected to chicken or yeast DNA (not shown). The hNKG2A probe did not yield detectable bands in the rat, although bands were clearly present for dog and cow DNA. The hNKG2C probe did not produce detectable bands except with human and monkey DNA (Fig. 3). Under conditions of low stringency, the hNKG2D probe, containing the entire open reading frame, hybridized to only one band in most digests, indicating homology within only a short segment of genomic DNA (not shown). We have recently cloned and sequenced this rat NKG2D homologue, which is 60% identical to human NKG2D at the amino acid level (S. Berg et al., manuscript in preparation).

Mapping of the Rat NKC and Localization to Chromosome 4. To map the NKC, Southern blots of PVG and DA liver DNA digested with 40 different restriction enzymes were hybridized to probes for genes encoded within or near to the human or mouse NKC. Informative RFLPs were found for a number of genes, including rNKR-P1B, rLy-49.9, rLy-49.12, and the previously undefined rat homologues of mLy-49C and hNKG2D (Fig. 4).

RFLP patterns were then investigated on liver DNA extracted from 223 (DA × PVG)F_1 × DA rats (for some RFLPs, only 85 animals). By this analysis, the members of the rat NKC were closely linked; hNKG2D, rNKR-P1B, and all of the mouse and rat Ly-49 RFLPs cosegregated in all but one of 223 rats (Fig. 5). A single crossover was observed within the NKC, splitting the *Ly49* genes into a centromeric group associated with the NKR-P1 family and with rat NKG2D, while the remaining, telomeric *Ly49* genes were associated with rat homologues of mLy-49C. This is similar to the mouse NKC, where mLy-49C maps telomeric to at least one other *Ly49* locus (44).

In the mouse, the NKC is linked to several unrelated genes, including *Kap* and *Igkc* (56). RFLP analysis revealed a similar linkage in the rat. A single recombination occurred between *Kap* and *Ly49*, placing *Kap* on the telomeric side of the NKC. The NKC also lay ~39.5 cM from *Igkc*, 7.1 cM from *Cd9*, and 9.5 cM from *Pthlh*. The *Igkc* and *Pthlh* loci have previously been mapped to rat chromosome 4 (57). Earlier karyotyping studies have shown considerable G-banding similarities between mouse chromosome 6 and rat chromosome 4 (57). The orientation of mouse and rat loci within these regions is therefore likely

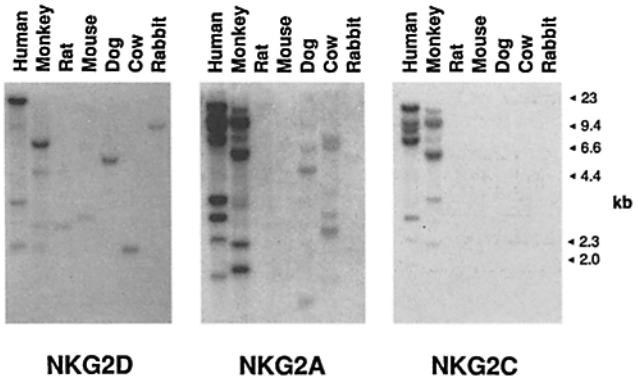


Figure 3. Search for NKG2 homologues in the rat by Southern blot analysis. DNA from several mammal species were digested with EcoRI, separated by electrophoresis, transferred to nylon membrane, and hybridized under low stringency conditions to cDNA probes for hNKG2A, -C and -D. Lane *1*, human; lane *2*, monkey; lane *3*, rat; lane *4*, mouse; lane *5*, dog; lane *6*, cow; lane 7, rabbit. The NKG2D probe hybridized to a band in the rat, whereas the -A and -C probes did not hybridize detectably above background. Approximate molecular weights in kb are indicated.

Figure 2. (*A*) rLy-49.9, -12 and -29 are type II integral membrane proteins with extracellular C-type lectin domains. For comparison, the mLy-49A sequence (41) is shown, the amino acid sequences are divided into six putative exon sequences as predicted by the genomic structure of mLy-49A (66). The transmembrane region is underlined and sites for *N*-glycosylation are boxed. The residues defining putative inhibitory G-protein (G_i) binding sites are underlined twice. (*B*) Per cent identical residues between rat and mouse Ly-49 sequences at the nucleotide/amino acid levels. (*C*) Dendrograms comparing the amino acid sequences of the putative cytoplasmic domains (exon 2) and amino-terminal exon encoding the lectin domain (exon 5) of the three rat sequences with eight mouse Ly-49 proteins (16, 17) (mLy-49G corresponding to -49G2 in reference 16 and mLy-49H renamed as announced by the authors in reference 17). rLy-49.9 is an 1193 bp cDNA encoding a 275–amino acid protein with a predicted molecular weight of 31,889. The consensus rLy-49.12 cDNA is a 1163–bp cDNA encoding a 265–amino acid protein with a predicted molecular weight of 31,215. rLy-49.19 is a1152-bp cDNA pseudogene encoding a truncated peptide of only 31 amino acids (not shown). When compared with the consensus sequences of the other rat Ly-49 cDNAs, the rLy-49.19 sequence contains a single-base insertion (G) at nt 210, deletion of an 8-nt consensus segment (ATTTTTCA) between bases 333 and 334, and a single base substitution (G to A) producing a stop codon at nt 492 (not shown). The 919-bp rLy-49.29 cDNA encodes a 269–amino acid protein with a molecular weight of 31,140. Nucleotide sequences are available from GenBank (accession numbers rLy-49.9: U56863; rLy-49.12 (consensus sequence): U56822; rLy-49.19: U56823; rLy-49.29: U56824).

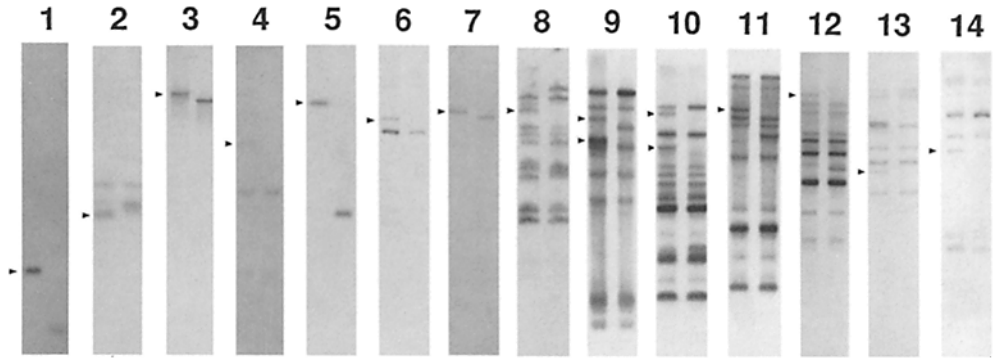


Figure 4. Southern blot RFLP analyses of liver DNA from PVG and DA rats hybridized to the probes indicated below (restriction enzymes in parenthesis). Left lanes, PVG; right lanes, DA. *Panel 1*, Ig κ C-region (HincII); *panel 2*, CD9 (HaeIII) *panel 3*, KAP (KpnI); *panel 4*, PTHLH (BstXI); *panel 5*, P1B (HindIII); *panel 6*, 5E6-5′ (EcoRV); *panel 7*, hNKG2D (BstXI); *panel 8*, rLy49.9 (EcoO-109I); *panel 9*, rLy49.12 (BstXI); *panel 10*, rLy49.12 (EcoO109I); *panel 11*, rLy49.12 (EcoRI); *panel 12*, rLy49.12 (HindIII); *panel 13*, 5E6-5′ (BstXI); *panel 14*, 5E6-5′ (EcoRI). The PVG-specific bands are indicated with arrows. Because of crosshybridization between Ly-49 genes, Ly-49 RFLPs need not have been specific for the particular genes used as probes. For simplicity, the Ly-49 RFLPs were provisionally termed Ly49-R1 (panel *12*), -R2 and -R3 (panel *9*, short and long bands, respectively), -R4 (panel *11*), -R5 and -R6 (panel *10*, short and long bands, respectively), -R7 (panel *8*), -R8 (panel *13*), -R9 (panel *14*), and -R10 (panel *6*).

to be similar, giving the following orientation: centromere–*Igkc–Cd9*–NKC–*Kap–Pthlh*. Our findings thus localize and orient the rat NKC within rat chromosome 4 and demonstrate conserved linkage of a large chromosomal region (~50 cM) between the mouse and the rat (Fig. 6 *A*).

PFGE Mapping of the NKC. To further map the loci within the NKC, we prepared PFGE Southern blots of rat DNA. PFGE of SalI- and SfiI-digested B cell DNA revealed fragments that spanned large parts of the NKC, some of which overlapped. By sequentially hybridizing these blots to several NKC probes and assembling the overlapping fragments, a tentative map of the rat NKC could be drawn (Fig. 6 *B*). We were not able to generate probes specific for each of the Ly-49 members, but two Ly-49 probes (49-CTM and 5E6-5′) did not cohybridize, except to very long fragments, indicating that they identify distinct chromosomal regions. The 49-CTM and hNKG2D probes cohybridized to several fragments, the shortest of which (XhoI, not shown) constrained the region containing NKG2D and at least one Ly-49 gene to less than 250 kb. Probes for rNKR-P1A and rNKR-P1B hybridized exclusively to a single 120-kb ClaI fragment, demonstrating close proximity of these genes. It was not clear from our data whether NKG2D was flanked by Ly-49 genes on both sides or just on the telomeric side, as schematically drawn in Fig. 6 *B*.

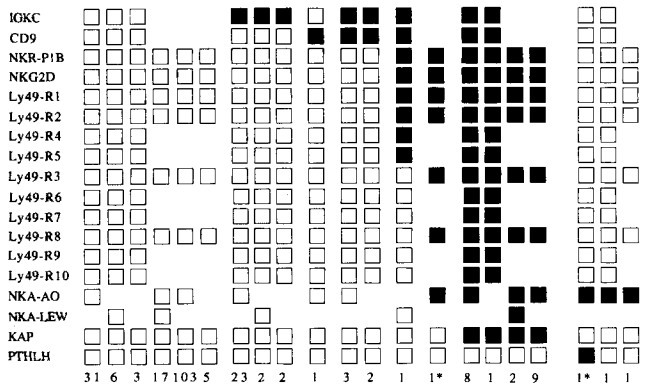


Figure 5. Linkage analysis of *Nka* and other loci on rat chromosome 4. 223 (DA × PVG)F_1 × DA rats were genotyped as either homozygous (DA/DA) or heterozygous (DA/PVG) at the indicated loci by RFLP analysis of liver DNA. Rats are displayed in different groups according to the typed loci and the location of crossovers. The *Nka* phenotypes of 210 of these animals were determined, either towards AO (n = 182), LEW (n = 9), or both AO and LEW (n = 19) donor lymphocytes (Fig. 1 *B*, LEW data not shown). A high alloreactivity phenotype was attributed to heterozygosity at *Nka*. The rLy-49.9 and rLy-49.12 probes crosshybridized extensively to other Ly-49 related genes. Therefore, several Ly-49 RFLPs were applied, to detect and localize as many members of the Ly-49 gene family as possible. Two rats showing high NK alloreactivity were homozygous at the surrounding parts of the chromosome (the two far right columns). These results, which were noninformative for mapping of *Nka*, could stem from double crossover, gene conversion or false positive alloreactivity results. The two columns marked with an asterisk represented conflicting results regarding the order of *Nka* and *Kap*.

Nka Is Located within the Rat NKC. Of the 223 rats used for RFLP analysis, 201 had been typed for in vivo NK alloreactivity toward donor TDL from AO rats, and 28 had been tested for alloreactivity toward TDL from LEW donors (including 19 that were tested for both). Cosegregation between *Nka* and the NKC genes was found for 198 out of the 201 rats typed with AO donor TDL (Fig. 1 *B*). The three discordant rats probably reflected double crossover or gene conversion, or were false positives (Fig. 5, the three far right columns). The single rat with a recombination inside the Ly-49 region (injected with LEW donor cells) demonstrated the high alloreactivity (PVG) *Nka* phenotype, being homozygous (DA/DA) centromeric to the crossover, and heterozygous (PVG/DA) on the telomeric side (Fig. 5). This crossover placed *Nka* telomeric to the region containing the centromeric portion of the Ly-49 gene family. It also localized *Nka* to a chromosomal region distinct from *Nkrp1b* and *Nkg2d*. A second crossover, with low alloreactivity, placed *Nka* centromeric to *Kap*. Thus, from these two crossovers *Nka* lay between the centromeric portion of the Ly-49 family and *Kap*. A third crossover, with high alloreactivity, conflicted with this result, placing Nka telomeric to *Kap* (Fig. 5). The findings with this rat, however, were discounted because injection was

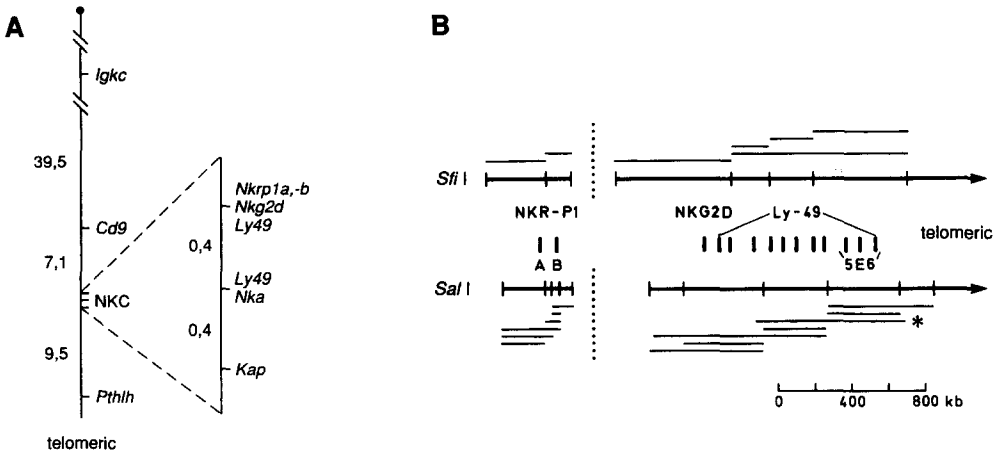


Figure 6. (*A*) Linkage map of rat chromosome 4 based on segregation of alleles in 223 backcrossed rats. Map distances are shown in cM. The localization of *Nka* was based on data from both AO and LEW donor cells. (*B*) Physical map of the rat NKC. PFGE Southern blots were generated with several rare-cutting restriction enzymes and sequentially hybridized to probes for NKC genes. Incomplete SalI and SfiI digests produced a number of partially overlapping fragments. The 49-CTM, 5E6-5′ and hNKG2D probes were used for the telomeric part of the complex. 49-CTM did not hybridize to the two most telomeric SalI- fragments. 5E6-5′, however, hybridized to these bands but not to the four most centromeric bands. The subgroup of Ly-49 genes related to mLy-49C (5E6) are indicated at the telomeric end. One 810-kb band, marked with an asterisk, hybridized to both probes. Two of the overlapping bands summed up to 740 kb, leaving 70 kb that could not be incorporated precisely. The centromeric/telomeric direction of the NKR-P1 segment was not known. The number of genes in the Ly-49 family is unknown, therefore the Ly-49 genes were drawn schematically. The NKR-P1A and P1B probes both hybridized to a single 120-kb ClaI fragment, and the hNKG2D and 49- CTM probes cohybridized to a 250-kb fragment with XhoI (not shown).

associated with extravasation of cells, which would result in reduced uptake of labeled donor cells, mimicking allorejection (high reactivity). We thus favor the interpretation supplied by the first two crossovers, which constrain *Nka* to a region of ~0.4 cM centromeric to *Kap*, containing the telomeric group of Ly-49 genes. This association of *Nka* with the Ly-49 gene family was further supported by studies relating *Nka* to the expression of Ly-49 transcripts.

Association of NK Alloreactivity with the Expression of Ly-49 Transcripts. The genetic linkage between *Nka* and the Ly-49 family of receptors led us to consider the hypothesis that NK-mediated lysis of allogeneic targets may be mediated by members of the Ly-49 family. Although mLy-49A mediates inhibition of natural killing, it binds to selective class I MHC antigens (13, 25, 26). Thus, it is capable of allorecognition, raising the possibility that other members of the Ly-49 family, particularly in the rat, might activate natural killing of allogeneic targets.

To further examine a possible association between *Nka* and *Ly49*, Northern blots were prepared from IL-2-activated NK cells derived from DA, PVG, (DA × PVG)F_1, and the MHC-congenic strain PVG.1AV1, which carries the DA MHC haplotype ($RT1^{av1}$) on a PVG background. NK cells from all strains expressed transcripts for NKR-P1, although levels in DA NK cells were somewhat higher than in the other strains tested (Fig. 7). In contrast, as assessed by hybridization to probes for rLy-49.9, rLy-49.12, rLy-49.19, rLy-49.29, mLy-49A, and mLy-49C, the level of transcripts for members of the Ly-49 family was markedly reduced in NK cells from the nonresponder DA strain compared with NK cells from alloreactivity-competent PVG, (DA × PVG)F_1 and PVG.1AV1 rats (6). Similar to *Nka*, the higher expression of Ly-49 genes in PVG appears to be a dominant trait, as deduced from the high level of expression in (DA × PVG)F_1 cells. The low expression of Ly-49 genes in DA NK cells ($RT1^{av1}$) apparently did not result from MHC-controlled repertoire selection, since PVG.1AV1 cells ($RT1^{av1}$) expressed levels of Ly-49 that were similar to those in PVG cells (Fig. 7). These findings

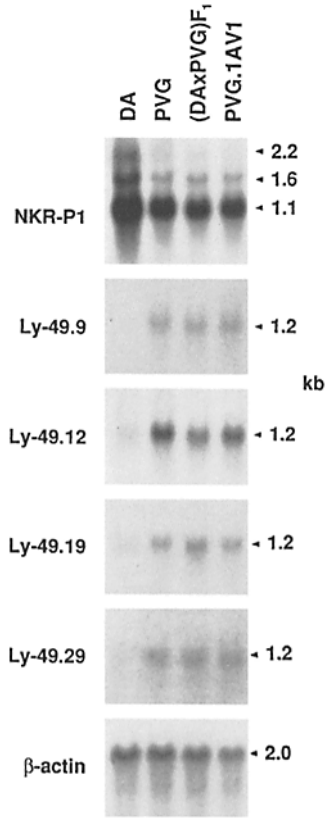


Figure 7. Northern blot analysis of IL-2-activated NK cells derived from DA, PVG, (DA × PVG)F_1 or PVG.1AV1 rats. The same pattern of low expression of Ly-49 genes in DA was also found with two mouse Ly-49 cDNA probes (mLy-49A and mLy-49C, not shown). Approximate molecular weights are indicated.

suggest that *Nka* may be a member of the Ly-49 family or represents a regulatory element controlling transcription of Ly-49 genes.

Discussion

In these studies, we have (*a*) identified a rat NKC region containing members of the NKR-P1 and Ly-49 families as well as a rat NKG2D homologue; (*b*) mapped the rat NKC to the telomeric part of chromosome 4; (*c*) demonstrated that the alloreactivity mediated by rat NK cells is controlled by a genetic locus, *Nka*, that lies within the NKC where it colocalizes with the telomeric group of *Ly49* loci and is distinct from *Nkrp1* and *Nkg2d*; (*d*) cloned four rat Ly-49 cDNAs and demonstrated considerable heterogeneity within their cytoplasmic domains; and (*e*) shown that the high responder *Nka* phenotype correlates with increased expression of Ly-49 transcripts.

All the NKLLR probes tested by Southern blotting mapped to the rat NKC. Although the many bands obtained with the Ly-49 probes indicate that there are several more rat Ly-49 genes, no Ly-49 related RFLPs mapped outside the NKC. This suggests that all Ly-49 genes reside in the complex, clustered within a region of 400–1,100 kb. NKG2 genes have previously not been described in rodents. hNKG2D is sufficiently distantly related to other NKG2 genes to merit classification as a separate group of NKLLRs, e.g., the COOH-terminal part of its carbohydrate recognition domain (CRD) is more similar to the CRDs of NKR-P1 than to other NKG2 molecules (58, S. Berg et al., manuscript in preparation). Its presence, therefore, does not suggest the existence of rat homologues to other NKG2 genes.

Our studies demonstrate the presence within the NKC of a locus, *Nka*, controlling NK-mediated alloreactivity. Similar to *Nka*, NK cell-mediated resistance to murine cytomegalovirus infection in the mouse is encoded by a locus (*Cmv1*) that maps to the NKC region on mouse chromosome 6 (59). Also, a correlation between NK cell alloreactivity and NK1.1 (mNKR-P1C) expression in crosses of inbred mouse strains has previously been described (60).

The nature of the *Nka* locus is not known. However, although DA NK cells are deficient in the lysis of allogeneic blasts, they are competent in the lysis of tumor targets (61), indicating that *Nka* influences target-specific interactions rather than nonspecific steps in the lytic pathway. This is supported by the finding that *Nka* maps to the NKC, which encodes families of lectin-like receptors. As in the mouse, rat alloreactivity is directed against MHC encoded gene products (2, 6). Our hypothesis is therefore that *Nka* encodes or regulates an allele-specific receptor for class I MHC molecules. In the mouse, at least one member of the Ly-49 receptor family, Ly-49A, binds to specific target class I MHC antigens. It is therefore particularly noteworthy that *Nka* is linked to the genes encoding the Ly-49 family of receptors and that the activity of *Nka* correlates with levels of Ly-49 expression.

The striking correlation between levels of Ly-49 expression and alloreactivity in DA, PVG, (DA × PVG)F_1, and PVG.1AV1 NK cells does not prove a causal relationship. In particular, we have only investigated expression in total NK cell populations. Furthermore, there is recent evidence that immature NK cells cultured from mouse fetal liver do not express Ly-49 and do not exhibit alloreactivity, but kill tumor targets (62). Their phenotypic similarity to DA NK cells raises the possibility that the lack of alloreactivity and the low Ly-49 expression are mutually independent features of DA NK cells with a common cause, e.g., blocked or erratic NK cell maturation. This would imply, however, that *Nka* controls NK cell maturation. Given the genomic localization of *Nka* in the Ly-49 region of the NKC, we consider it more likely that *Nka* encodes or regulates the expression of Ly-49 molecules or related as yet undefined NKLLR members.

The interaction between mouse Ly-49A and cognate MHC inhibits natural killing (13), in accord with the "missing self" hypothesis, that natural killing is inhibited by target cell MHC class I. In contrast, the high responder Nka^{PVG} phenotype is dominant in $Nka^{PVG/DA}$ heterozygotes, demonstrating that this phenotype is not due to the loss of an inhibitory (DA) allele, but to the inheritance of an allele (PVG) promoting alloreactivity. The possibility that the PVG allele of *Nka* represents a dominant negative mutation, somehow blocking an inhibitory receptor function, cannot be entirely dismissed, but does not explain the correlation between alloreactivity and Ly-49 expression. In spite of the weak signals obtained from DA NK cells on Northern blots, we cannot exclude normal expression of a single or very few Ly-49 genes. However, the probes used crosshybridized extensively to other Ly-49 genes. Moreover, DA rats are deficient in NK-mediated alloreactivity to lymphocytes from a large panel of donor strains (52). We therefore consider it unlikely that the inability of DA NK cells to lyse allogeneic targets reflects the activity of inhibitory Ly-49 molecules. The most likely interpretation of our observations is therefore that Ly-49 members or other coregulated, as yet undefined NKLLR molecules encoded in the same region of the rat NKC activate rather than inhibit natural killing.

Several studies indicate that NK cells can be activated, rather than inhibited by MHC encoded structures. In cold target inhibition experiments with PVG NK cells, blasts from the LEW.1LM1 strain, which is genetically identical to LEW except for a 100-kb homozygous chromosomal deletion in the *RT1.C* region of the MHC, failed to specifically inhibit lysis of LEW blasts (6). Similarly, NK cells in irradiated C57BL/6 mice recognize and eliminate hematopoietic cells from H-2D^d transgenic C57BL/6 donors (63). In the human, recent studies have further demonstrated that some NK clones can be activated rather than inhibited by the selective expression of MHC antigens on target cells (64, J. Gumperz, Stanford, personal communication). These observations suggest the existence of an activating NK cell receptor for MHC gene products.

PFGE analysis of the DA and PVG NKC regions failed to detect insertion/deletion patterns, and Ly-49 probes de-

tected similar numbers of crosshybridizing bands on Southern blots from PVG and DA (not shown). Thus, the low Ly-49 expression in DA LAK cells did not result from a chromosomal deletion of *Ly49* loci in DA. Neither could it be explained by MHC-controlled shaping of the Ly-49 repertoire or by the recently proposed "receptor calibration" mechanism (29), as (DA × PVG)F_1 and PVG.1AV1 NK cells showed high Ly-49 expression.

We instead favor the hypothesis that the deficient alloreactivity is caused by a lack of activating alloreceptors, and suggest the possibility that NKLLR members encoded in the Ly-49 region of the NKC transmit activation signals upon binding to non-self class I MHC gene products. Structural analysis of the rLy-49 cDNAs supports the idea that different Ly-49 molecules may transduce divergent signals. For example, the extracellular lectin-like domains of rLy-49.9 and rLy-49.29 proteins are nearly identical, suggesting a common ligand. Their cytoplasmic domains, in contrast, are only 63% identical. Although little is known about signal transduction through Ly-49, the divergence of the rat Ly-49 cytoplasmic domains seems consistent with the hypothesis that distinct Ly-49 proteins deliver different transmembrane signals to NK cells. Similarly, recent evidence suggests that CD94, a lectin expressed on human NK cells, can elicit both positive and negative signals to NK cells (65). An analogous example is provided by the recently identified p50 molecules, putative activating receptors among the KIR family of human NK cell receptors for MHC gene products (64). In conclusion, our studies define a new genetic locus that controls NK-mediated lysis of allogeneic cells, and they suggest that it may be mediated by NKLLR molecules, possibly members of the Ly-49 family.

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