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MHC class I alloantigen specificity of Ly-49⁺ IL-2-activated natural killer cells

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The molecular basis of target cell recognition by CD3⁺ natural killer (NK) cells is poorly understood, despite the ability of NK cells to lyse specific tumour cells. In general, target cell major histocompatibility complex (MHC) class I antigen expression correlates with resistance to NK cell-mediated lysis, possibly because NK cell-surface molecules engage MHC class I antigens and consequently deliver inhibitory signals. Natural killer cell allospecificty involves the MHC class I peptide-binding cleft, and further understanding of this allospecificity should provide insight into the molecular mechanisms of NK cell recognition. The Ly-49 cell-surface molecule is expressed by 20% of CD3⁺ NK cells in C57BL/6 mice (H-2b). Here we show that C57BL/6-derived, interleukin-2-activated NK cells expressing Ly-49 do not lyse target cells displaying H-2a or H-2b despite efficient spontaneous lysis by Ly-49⁺ effector cells. This preferential resistance correlates with expression of target cell MHC class I antigens. Transfection and expression of H-2Db, but not H-2Kb or H-2Lb, renders a susceptible target (H-2b) resistant to Ly-49⁺ effector cells. The transfected resistance is abrogated by monoclonal antibodies directed against Ly-49 or the α1/α2 domains of H-2Db, suggesting that Ly-49 specifically interacts with the peptide-binding domains of the MHC class I alloantigen, H-2Db. Inasmuch as Ly-49⁺ effector cells cannot be stimulated to lyse H-2Db targets, our results indicate that NK cells may possess inhibitory receptors that specifically recognize MHC class I antigens.

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As Ly-49 seems to be encoded by a multigene family \(^{10}\) in the mouse NK complex \(^{9}\), we have speculated that Ly-49 may be representative of a family of NK cell surface molecules that recognize polymorphic ligands \(^{14}\). To address this possibility, we prepared Ly-49\(^{+}\) and Ly-49\(^{-}\) effector cells and determined their target cell specificity. Except for Ly-49 expression, the CD3\(^{+}\) effector cell subsets were equivalent with respect to expression of NK cell surface molecules (Fig. 1) and ability to lyse certain targets (Table 1).

The Ly-49\(^{+}\) effector cells, however, could not lyse spontaneously a panel of other tumour cells that were lysed by Ly-49\(^{-}\) effector cells (Table 1). The preferentially resistant targets could not be lysed by Ly-49\(^{+}\) effector cells even by other mechanisms (such as antibody-dependent cytotoxicity, anti-NK1.1 monoclonal antibody-induced redirected lysis \(^{34}\) or lectin-induced lysis (data not shown). Preferential target cell resistance correlated with target cell H-2 haplotype (Table 1). Target cells homozygous or heterozygous (with H-2\(^{b}\)) for the H-2\(^{b}\) or H-2\(^{b}\) haplotypes were resistant to lysis by Ly-49\(^{+}\) effector cells. The only exceptions to this correlation were YAC-1 cells (H-2\(^{b}\)), including H-2K\(^{b}\), H-2D\(^{b}\), which were very sensitive to lysis by effector cells, and WEHI-3 and Bl88 (H-2\(^{b}\)) cells, which were resistant to both cell subsets. Other factors may influence the susceptibility of these exceptional targets to both Ly-49\(^{+}\) and Ly-49\(^{-}\) effector cells. These results suggested that an MHC antigen of the H-2\(^{b}\) or H-2\(^{b}\) haplotype is a target cell ligand for Ly-49. Furthermore, R1.1 cells (H-2\(^{b}\)) were preferentially resistant to lysis by Ly-49\(^{+}\) effector cells but derivative R1E/TLL8.1 cells, which do not express MHC class I antigens (B\(^2\)-microglobulin gene mutation \(^{15}\)), were susceptible to lysis by both effector cell subsets (Table 1). The H-2\(^{b}\)-derived cell line LBRM-33-1A5 was susceptible to lysis by both effector cell subsets (Table 1) but it did not express MHC class I antigens. Thus, target cell surface expression of MHC class I antigens of certain haplotypes correlated with resistance to lysis by Ly-49\(^{+}\) effector cells.

To explore further the relation between specific MHC class I antigens and target cell resistance to Ly-49\(^{+}\) effector cells, we transfected the C1498 cell line (H-2\(^{b}\)), which is susceptible to lysis by Ly-49\(^{+}\) and Ly-49\(^{-}\) effector cells (Table 1), with complementary DNAs encoding H-2\(^{b}\), H-2\(^{k}\), H-2L\(^{b}\), or with empty vector DNA (Fig. 2A). Only transfected C1498 cells expressing H-2\(^{b}\) became resistant to lysis by Ly-49\(^{+}\) interleukin-2 (IL-2)-activated NK cells (Fig. 2B). These transfectants remained susceptible to spontaneous lysis by Ly-49\(^{+}\) effector cells, indicating that the gene-transferred resistant phenotype was specific for Ly-49\(^{+}\) effector cells.

Transfected resistance to Ly-49\(^{+}\) IL-2-activated NK cell-mediated lysis was reversed by anti-Ly-49 monoclonal antibody, supporting a specific role for Ly-49 in recognizing H-2\(^{b}\) on target cells (Fig. 3A). As we used the F(ab\(^{\prime}\)) anti-Ly-49 antibody and as C1498 does not express Fc receptors, it is unlikely that anti-Ly-49 induced redirected lysis \(^{14}\). Moreover, anti-Ly-49 does not induce lysis of Daudi cells which are susceptible to anti-NK1.1 antibody-induced lysis by these effector cells (data not shown; ref. 14). Blocking studies with anti-H-2\(^{b}\)-specific antibodies (Fig. 3B) demonstrated that an anti-H-2\(^{b}\)-\(a\)/\(a\) domain-specific antibody allowed Ly-49\(^{+}\) NK cells to lyse the H-2\(^{b}\)-transfected target. In contrast, an anti-H-2\(^{b}\)-\(a\)/\(3\) domain-specific antibody did not affect lysis. Similar results
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FIG. 2. A Flow cytometric analysis of C1498 cells (H-2d) and C1498 cells transfected with H-2Dd, H-2Kd, H-2Ld or vector DNA alone. Cells were incubated with mAb 34-2-12S (anti-H-2d), solid lines; ref. 20, mAb SF1-1.11 (anti-H-2Kd), heavy dotted line; ref. 29, mAb 30-5-7S (anti-H-2Ld, dashed lines; ref. 28), or mAb AF6-88.5.3 (anti-H-2Kd, fine dotted line; ref. 30), followed by FITC-conjugated goat anti-mouse immunoglobulin. For clarity, background staining with FITC-conjugated second step reagent alone is not shown but was identical to the lowest level staining. Before any testing in cytotoxicity experiments, these cell lines were chosen from a larger panel of transfectants because of high levels of comparable expression of the transfected gene product. B. C1498 cells (H-2d) transfected with H-2Dd were preferentially resistant to lysis by Ly-49 positive effector cells whereas transfecion of the H-2Kd or H-2Ld DNA did not influence spontaneous killing by Ly-49 effector cells. For each DNA transfection, 3 independent cell lines were analysed: C1498-H-2Dd, D8 (a), D12 (b), D13 (c), C1498-H-2Kd, K4 (d), K13 (e), K18 (f); C1498-H-2Ld, L1 (g), L5 (h), L8 (i); as well as the following controls: C1498 V.1 (transfected with vector alone) (j), wild-type C1498 (k), and YAC-1 target cells (l). The cell lines assayed for spontaneous lysis by Ly-49 (filled circles) or Ly-49 (circles) effector cells.

METHODS. The H-2Dd DNA was obtained by amplification by polymerase chain reaction (PCR) of cDNA derived from DBA/2 mouse liver with primers specific for the published H-2Dd gene product. Sequence analysis of the H-2Dd cDNA showed identity with published sequences except for a single codon (Arg 218 for Gin 218) alteration in the α2 domain, and a deletion (amino acids 316-340) in the cytoplasmic tail (data not shown). The cDNA was directionally cloned behind the SV40 promoter in the pcEXV3 vector3, producing pD9V.1. Cells transfected with this construct display epitopes recognized by all H-2Dd specific mAbs and can present antigen to an H-2Dd-restricted T cell. The H-2Ld cDNA also was amplified by PCR, sequenced and directionally cloned behind the human β-actin promoter in the pSV2-neo vector5, producing pl444. Expression and function of transfected H-2Ld was also verified. The pH-2SV33 plasmid contains the H-2Kd cDNA behind the SV40 early promoter5 and was provided by P. Kourilsky. 5×10⁵ C1498 cells were transfected by electroporation (Bio-Rad gene pulser with 580 V, 25 μF in phosphate-buffered saline, 0.4-cm gap) with EcoRV-digested pcDNA4 (10 μg) alone, or cotransfected with HindIII-digested plasmid pDV.1 (15 μg). EcoRV-digested pH-2SV33 plasmid (15 μg) and HindIII-digested empty vector pCEV, plasmid (15 μg) together with HindIII- digested pSV2-neo (4 μg). Cells were seeded in 96-well plates at 2.5×10⁴ cells per well and selected with G418 (GBCO) 1.5 μg ml⁻¹ in RPMI containing 20% FCS. Growing cells from individual wells were expanded and examined by flow cytometry with mAbs specific for private epitopes on the endogenous and transfected gene products. For each plasmid, G418-resistant cells grew in <50% of seeded wells, suggesting clonality. This was confirmed by the uniform level of expression of the transfected cDNA product by flow cytometric analysis (not shown). Cell lines expressing comparable amounts of the transfected and endogenous class I MHC genes were selected for further analysis. Cr-labelled target cells were incubated with the indicated effector cells at varying E:T ratios as described in Table 1. The only discordance in susceptibility to killing by the effector cell subsets was observed in the C1498 target cell transfected with the H-2Dd gene. (Some variation was observed in the extent to which individual transfecants were lysed by both Ly-49 and Ly-49 IL-2-activated NK cells. This presumably was due to clonal variability in other factors affecting susceptibility to IL-2-activated NK cell-mediated lysis.)

FIG. 3. A Resistance to Ly-49- IL-2-activated NK cells transfected with the H-2Dd gene is specific for Ly-49. Cytotoxicity by Ly-49 effector cells against C1498-H-2Dd, D12 (a), D13 (b), C1498-H-2Kd, K13 (c), C1498-H-2Ld, L5 (d) or C1498 V.1 (e) in the presence (filled circles) or absence (circles) of IFN-γ anti-Ly-49 mAb (A1) is shown. Incubation with the anti-Ly-49 mAb enabled Ly-49 effector cells to lyse transfected resistant tumour cells to a level that was comparable to spontaneous lysis of the transfected susceptible targets. This was also comparable to lysis of the same targets by Ly-49 effector cells (not shown). The effect of mAb A1 was specific for Ly-49 effector cells. An isotype-matched control mAb did not affect lysis of these targets by Ly-49 effector cells and mAb A1 did not affect lysis of any targets by Ly-49 effector cells (not shown). Similar results with mAb A1 were observed for all targets that were preferentially resistant to Ly-49 effector cells (not shown). The functional effects of mAb A1 were not due to redirected lysis, a mechanism requiring that an Fc receptor on the target bind to the Fc portion of the stimulating antibody4,14. First, mAb A1 allowed Ly-49 effector cells to lyse resistant target cells that do not express Fc receptors (C1498-H-2Dd cell lines, and L5178Y-R.1). Second, Fab or F(ab')2 fragments of mAb A1 were as effective as the intact antibody in allowing lysis. Third, Daudi cells, which are susceptible to mAb-induced redirected lysis by Ly-49 NK cells, using mAbs directed against the NK1.1, VEA, and Ly-5 antibodies (see above), were not lysed by Ly-49 NK cells incubated with mAb A1 (not shown). B. The...
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TABLE 1  Lysis of murine tumour cells by Ly-49<sup>+</sup> and Ly-49<sup>−</sup> IL-2-activated NK cells

<table>
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<tr>
<th>Cell line</th>
<th>Type</th>
<th>H-2&lt;sup&gt;−&lt;/sup&gt; (n)</th>
<th>% Specific cytotoxicity by Ly-49&lt;sup&gt;+&lt;/sup&gt; NK cells</th>
<th>% Specific cytotoxicity by Ly-49&lt;sup&gt;−&lt;/sup&gt; NK cells</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>25</td>
<td>12.5</td>
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<td>YAC-1</td>
<td>T cell</td>
<td>a (17)</td>
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Lysis of target cells was determined in triplicate at the indicated effector:target (E:T) cell ratios (25:1, 12.5:1, and 2.5:1) in standard 51Cr-release cytoxicity assays at 37°C for 4 h with day-9 IL-2-activated NK cells against the indicated tumour cells. Results are expressed as per cent specific cytotoxicity as described<sup>4</sup>. To obtain enough effector cells and to avoid any functional effect produced by anti-Ly-49 antibody based separation, we prepared Ly-49<sup>+</sup> and Ly-49<sup>−</sup> effector cells from IL-2-activated NK cells as detailed in Fig. 1 legend. (As much as monoclonal antibody A1 (anti-Ly-49<sup>+</sup> ref. 21) was required to prepare the NK cell subsets, and as this antibody had functional effects on Ly-49<sup>+</sup> effector cells (see later), results were uninterpretable if these effector cell subsets were prepared from freshly isolated NK cells.) The NK cell subsets were prepared in 17 independent experiments. In every experiment, lysis of the prototypical murine NK cell-sensitive target, YAC-1, was assayed. Results were comparable in each experiment, showing that both effector cell subsets lyse this target equivalently. Other targets were tested for lysis by the effector cell subsets in n experiments; results were comparable but are shown for only one representative experiment so that the data could be organized with respect to target-cell resistance and H-2 haplotypes. Targets were obtained from the American Type Culture Collection; origins of the cell lines are referenced in their catalogue. The cell type and H-2 haplotype were verified by examination of primary references and by flow cytometry using monoclonal antibodies specific for private epitopes on H-2 molecules. R1E/T8L.1 (ref. 15) and LBRM-33-1A5 lack MHC class I expression by flow cytometric analysis with the anti-H-2 class antibody M1/4239.8 (ref. 22) data not shown). The absence of class I MHC expression on LBRM-33-1A5 cells has not been reported previously. Some targets (for example, YAC-1, 1C-21, B16S and C1498) were killed equally well by both effector cell subsets and others (such as B8B8 and WEHI-3) were not killed by either subset. Interestingly, several targets were resistant to Ly-49<sup>−</sup> effector cells. Targets preferentially resistant to Ly-49<sup>+</sup> effector cells could not be lysed by these effector cells even by the mechanism of antibody-dependent cellular cytotoxicity (ADCC; not shown). Moreover, resistant targets that expressed Fc receptors could not be killed by redirected lysis with antibodies directed against the NK1.1, VEA, or Ly-6 antigens<sup>14</sup> or by lectin (concanavalin A)-induced lysis (not shown), even though Ly-49<sup>+</sup> and Ly-49<sup>−</sup> effector cells were equivalent with respect to their ability to mediate ADCC, monoclonal antibody-induced redirected lysis, or lectin-induced lysis of the human target, Daudi (data not shown).

α1/α2-domains of H-2<sup>D</sup> are involved in the H-2<sup>D</sup> gene-transferred resistance to Ly-49<sup>+</sup> effector cells. Fab<sub>α</sub> fragments of the indicated mAbs were incubated with the Ly-49<sup>+</sup> or Ly-49<sup>−</sup> effector cells and the C1498.D12 target cell.

alpha<sub>d</sub>OS. Ly-49<sup>+</sup> IL-2-activated NK cells were prepared and used in cytotoxicity experiments at varying E:T ratios as described in Table I. The mAbs were affinity purified by protein A-Sepharose chromatography according to standard procedures. Fab<sub>α</sub> fragments were generated by pepsin digestion and then depleted of undigested mAb and Fc fragments by passage over a protein A-Sepharose column. Purity was determined by 7% SDS-PAGE and biological activity was confirmed by flow cytometry. The purified mAbs and their Fab<sub>α</sub> fragments bound to the effector cells equivalently. Fab<sub>α</sub> fragments of mAbs A1, 34-5-8S (anti-H-2<sup>D</sup>α1/α2; ref. 28) and 34-2-12S (anti-α3; ref. 28) were added to the cytotoxicity assay at 25 μg ml<sup>−1</sup>; 25:1 E:T ratio, as indicated. Fab<sub>α</sub> fragments of the mAbs were used to avoid the possibility of redirected lysis (anti-Ly-49<sup>+</sup>) or ADCC (anti-H-2<sup>D</sup>α).
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Ly-49 is genetically linked to NK1.1 in the mouse NK complex. NK1.1 encodes an alloantigen molecule expressed by all murine NK cells and that activates, rather than inhibits, cytotoxicity. But the engagement of Ly-49 has a dominant negative effect over stimulation through NK1.1. Such negative effects are seen in B-cell signalling, where cross linking of FcγRII-B1 inhibits anti-immunoglobulin-induced stimulation. Thus, allospecificity of human CD3- NK cell clones may be regulated largely by inhibitory receptors, similar to Ly-49 (ref. 19), and their putative MHC ligands.10

Our blocking studies with monoclonal antibodies implicate the peptide binding domain of H-2D in MHC class I-associated target cell resistance to activated NK cells. This is consistent with transfection studies demonstrating that a single amino-acid mutation (residue 74) in the a helix of HLA-A2 converted target cells to an NK cell-resistant phenotype. Inasmuch as this alteration provides access to a side pocket in the peptide-binding cleft, MHC class I molecules may present peptides to NK cells as well as to T cells.

The physiological relevance of NK cell allospecificity remains to be determined. Molecular dissection of other allo-recognition phenomena, namely tissue rejection, and T-cell allospecificity, have provided insight into the normal physiological operations of these complex biological systems. Likewise, further investigation of NK cell allospecificity should help us understand NK cell recognition. This could explain an apparent paradox in our study; Ly-49 NK cells lyse syngeneic tumour cells but not normal syngeneic cells. Perhaps NK cell (inhibitory) allospecificity mimics NK cell recognition of self-MHC class I molecules bearing specific peptides that may be present in normal cells but absent in syngeneic tumour cells. Whatever the explanation, our results have implications for the regulation of T-cell and NK-cell activity in vivo because target cell MHC class I antigens seem to influence both types of cytotoxic lymphocytes but with opposite functional consequences. Finally, this raises the possibility that certain associations of MHC class I antigens with human diseases may be related to the effect of MHC class I molecules on NK cells.

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