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ANALYSIS OF AND ROLE FOR EFFECTOR AND TARGET CELL STRUCTURES IN THE REGULATION OF VIRUS INFECTIONS BY NATURAL KILLER CELLS

A Dissertation Presented

By

RANDY R. BRUTKIEWICZ

Submitted to the Faculty of the
University of Massachusetts Medical School in partial
fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN IMMUNOLOGY AND VIROLOGY
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RANDY R. BRUTKIEWICZ

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ABSTRACT

The overall emphasis in this thesis is the study of the regulation of virus infections by natural killer (NK) cells. In initial analyses, vaccinia virus (VV)-infected cells were found to be more sensitive to NK cell-mediated lysis during a discrete period of time post-infection. This enhanced susceptibility to lysis correlated with enhanced triggering (but not binding) of the effector cells and a concomitant decrease in target cell H-2 class I antigen expression. Furthermore, VV-infected cells became resistant to lysis by allospecific cytotoxic T lymphocytes (CTL) at a time when they were very sensitive to killing by NK cells or VV-specific CTL. This suggested that alterations in class I MHC antigens may affect target cell sensitivity to lysis by NK cells.

The hypothesis that viral peptide charging of H-2 class I molecules can modulate target cell sensitivity to NK cell-mediated lysis was tested by treating target cells with synthetic viral peptides corresponding to the natural or minimal immunodominant epitopes defined for virus-specific CTL, and then target cell susceptibility to NK cell-mediated lysis was assessed. None of the 12 synthetic viral peptides used were able to significantly alter target cell lysis by NK cells under any of the conditions tested.

In order to determine if H-2 class I molecules were required in the regulation of a virus infection by NK cells in $vivo$, intact or NK-depleted (treated with anti-asialo GM_1 antiserum) β_2-microglobulin-deficient [β_2m (-/-)] mice, which possess a defect in H-2 class I

antigen expression, were infected with the prototypic NK-sensitive virus, murine cytomegalovirus (MCMV). In anti-asialo GM_1-treated β_2m (-/-) mice, as well as in β_2m$^+$ (H-2 class I normal) control mice also treated with anti-asialo GM_1, a significant enhancement in splenic MCMV titers as compared to NK-intact animals, was observed. When thymocyte expression of H-2 class I molecules (H-2D^b) in normal mice was analyzed, it was found that following MCMV infection, H-2D^b expression was significantly greater than the low level of expression found in uninfected thymocytes. In marked contrast, thymocytes from β_2m (-/-) mice did not display any detectable H-2D^b before or after infection. These *in vivo* results demonstrate that NK cells can regulate a virus infection, at least in the case of MCMV, <u>independent</u> of H-2 class I molecule expression.

Thymocytes from uninfected normal mice were found to be very sensitive to NK cell-mediated lysis, whereas those from MCMV-infected animals were completely resistant, presumably due to the protective effects of MCMV-induced interferon (IFN). However, thymocytes from MCMV-infected β_2m (-/-) mice were only slightly protected from lysis by NK cells, consistent with the inverse correlation between MHC class I antigen expression and sensitivity to NK cell-mediated lysis. These results provide *in vivo* evidence suggesting a requirement for MHC class I molecules in IFN-mediated protection from lysis by NK cells.

In addition to the analysis of H-2 class I molecules on target cells, the identity of a molecule present on the surface of all NK cells

and other cytotoxic effector cells, which is recognized by a monoclonal antibody (mAb) generated in this laboratory designated CZ-1, and can also modulate NK cell triggering, was also of interest. This laboratory has previously reported that this antigen is upregulated on cytotoxic (and other) lymphocytes following a virus infection *in vivo*, or upon activation *in vitro*. Using competitive FACS analysis and fibroblasts transfected with various isoforms of CD45, it was found that mAb CZ-1 recognizes a sialic acid-dependent epitope associated with a subpopulation of CD45RB molecules.

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ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
β_2m	β_2-microglobulin
β_2m (-/-)	β_2m-deficient
C '	complement
CD45	leukocyte common antigen
CD45RA	antigen recognized by antibody specific for CD45 restricted exon A
CD45RB	antigen recognized by antibody specific for CD45 restricted exon B
CD45RC	antigen recognized by antibody specific for CD45 restricted exon C
Con A	concanavalin A
CTL	cytotoxic T lymphocyte(s)
DAG	diacylglycerol
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
H-2	murine major histocompatibility complex
HIV-1	human immunodeficiency virus-1
HLA	human leukocyte antigen(s)
HSV-1	herpes simplex virus type 1
i.o.	intraorbital
i.p.	intraperitoneal
ICAM	intercellular adhesion molecule
IFN(s)	interferon(s)

IL-2	interleukin-2
IP$_3$	inositol 1, 4, 5-trisphosphate
L(LCMV)	L929 cells persistently-infected with LCMV
LAK	lymphokine-activated killer
LCMV	lymphocytic choriomeningitis virus
LFA-1	leukocyte functional antigen-1
LFA-3	leukocyte functional antigen-3
LGL	large granular lymphocyte
MCMV	murine cytomegalovirus
MHC	major histocompatibility complex
MHV	mouse hepatitis virus
MOI	multiplicity of infection
N-CAM	neural cell adhesion molecule
NK	natural killer
NKR-P1	natural killer receptor protein-1
PE	phycoerythrin
PEC	peritoneal exudate cells
PFU	plaque-forming unit(s)
PI	phosphatidylinositol
PKC	protein kinase C
SA-PE	streptavidin-phycoerythrin
SCID	severe combined immunodeficiency
T200/0	Ψ2 cells transfected with the mouse CD45 gene not containing the restricted exons

T200/ABC	Ψ2 cells transfected with the mouse CD45 gene containing all 3 restricted exons
T200/BC	Ψ2 cells transfected with the mouse CD45 gene containing only the restricted exons B and C
T200/C	Ψ2 cells transfected with the mouse CD45 gene containing only the restricted exon C
TAP	transporter associated with antigen processing
TMEV	Theiler's murine encephalomyocarditis virus
TNFα	tumor necrosis factor-α
VSV	vesicular stomatitis virus
VV	vaccinia virus

CHAPTER I

INTRODUCTION

A. Overview of Natural Killer (NK) Cells

Discovery and initial characterization of natural killer cells. In
the early 1970s, a few groups studying the immune response to
tumors began to report on the ability of leukocytes from
nonimmunized animals (Herberman et al., 1973; Nunn et al., 1973;
Greenberg and Playfair, 1974) and humans (Takasugi et al., 1973) to
lyse various target cells. In these initial reports, it was not entirely
clear which effector cell(s) was mediating this cytolytic activity. For
example, one study (Herberman et al., 1973) was only able to
conclude that the effector cells were "not θ-bearing lymphocytes",
and therefore not T cells. Another report suggested that the effector
cell(s) mediating spontaneous cytotoxic reactivity against P815
mastocytoma targets was of "neither phagocytic or T-cell origin"
(Greenberg and Playfair, 1974). These initial studies stimulated an
interest to determine if, indeed, a novel naturally cytotoxic effector
cell capable of killing tumor cells existed. In investigations of cell-
mediated immune responses to tumor cells, in particular those
tumors induced by murine leukemia viruses, it was found that
leukocytes from unimmunized mice could "specifically" lyse leukemia
cells induced by Moloney leukemia virus and those induced by other
means which resulted in the expression of endogenous retroviral
antigens (Kiessling et al., 1975a; Herberman et al., 1975a). These two

groups were also able to demonstrate that the effector cell responsible for this lytic activity was a nonadherent (i.e., non-macrophage/monocyte), non-T, non-B lymphoid cell (Kiessling et al., 1975b; Herberman et al., 1975b). The results of these studies suggested that a new type of cytotoxic lymphoid cell existed and was given the name, "natural killer" (NK) cell, by Kiessling and his colleagues (Kiessling et al., 1975a, b). Following this initial characterization of NK cells, these and other groups went on to further show that NK cells are bone marrow-derived (Haller and Wigzell, 1977; Welsh, 1978), large granular lymphocytes (LGL; Timonen et al., 1979), which express receptors for the F_c portion of IgG (CD16; Herberman et al., 1977a), in addition to a low density of what was previously thought to be primarily a T cell-specific antigen in mice, Thy 1 (Herberman et al., 1978). It was also found that NK cells could be further distinguished on the basis of the expression of asialo GM_1 (Kasai et al., 1980) and NK1.1 (Glimcher et al., 1977) in mice, or CD56 (Lanier et al., 1989a) in humans. These last 3 markers, in addition to CD16, are used to identify and enrich for highly purified populations of NK cells.

Induction and augmentation of NK cell activity. A number of studies found that certain pathogenic agents, such as Bacille Calmette-Guérin (BCG), which is a strain of *Mycobacterium tuberculosis* (Wolfe et al., 1976), parasites such as *Toxoplasma* (Hauser et al., 1982) or *Trypanosoma* (Hatcher and Kuhn, 1982), or

viruses (Welsh and Zinkernagel, 1977; Herberman et al., 1977b; MacFarland et al., 1977; Wong et al., 1977) could induce NK cell activity. Furthermore, synthetic compounds, such as tilorone, poly I:C or pyran copolymer, could also enhance NK cell activity (Gidlund et al., 1978; Djeu et al., 1979). This was due, at least in part, to the ability of these agents to stimulate the production of α/β type interferons (IFNα/β), which are potent inducers of NK cells (Gidlund et al., 1978; Welsh, 1978; Djeu et al., 1979) and, like IFNγ, can stimulate NK cell blastogenesis *in vivo* (Biron et al., 1984). Additionally, it was found that a lymphokine important for T cell growth, interleukin 2 (IL-2), could augment the cytotoxic activity of NK cells (Henney et al., 1981), be used as a growth factor in the *in vitro* propagation of NK cells (Herberman, 1982), and induce the proliferation of NK cells *in vivo* (Biron et al., 1990). Furthermore, this growth factor has been used in clinical trials in which so-called lymphokine-activated killer (LAK) cells (Grimm et al., 1982) have been propagated *in vitro* with IL-2 and then adoptively transferred into tumor-bearing patients, as an attempt at tumor immunotherapy (Herberman et al., 1987). Therefore, many natural and synthetic substances have the capacity to significantly enhance the cytotoxic ability of NK cells and these, in some cases, have found their way into the clinic.

Steps involved in the cytolytic process by NK cells. As is the case for cytotoxic T lymphocytes (CTL), the killing of a target by NK

cells can be separated into at least 4 distinct steps as outlined below (Figure 1):

1. <u>Recognition and binding</u>: The recognition of and binding to a target by an NK cell is $[Mg^{2+}]$-dependent, but $[Ca^{2+}]$-independent (Hiserodt et al., 1982; Quan et al., 1982). This occurs via ill-defined receptors, but a number of adhesion molecules have been suggested to play a role in the interactions between an NK cell and its target. Many of these molecules have also been implicated in target cell recognition by T lymphocytes. For example, leukocyte functional antigen-1 (LFA-1) on the effector cells can use either intercellular adhesion molecule (ICAM)-1 or ICAM-2 as ligands on the targets (Springer, 1990). The interaction of NK (or T) cell CD2 with its ligand, LFA-3 (CD58) appears to be very important in signalling of the effector cells (Ritz et al., 1988; Bierer et al., 1989). It has recently been found that a marker predominantly found on human NK cells, CD56 (also known as Leu-19 or NKH-1), is actually neural cell adhesion molecule (N-CAM; Lanier et al., 1989a). Although mAb to CD56 have not been shown to inhibit either cytotoxicity or the binding of NK cells to their targets by themselves (Nitta et al., 1989; Lanier et al., 1991a), these mAb can synergize with mAb to LFA-1 and/or LFA-3 to block both reactions (Nitta et al., 1989). A number of other adhesion molecules, not necessarily restricted to NK cells, have been suggested to play a role in NK cell-mediated lysis (Kärre et al., 1991), but the importance of these molecules in the killing of targets by NK cells remains to be established.

1. Recognition and Binding

2. Triggering of NK cell by determinants on target

3. Release of cytotoxic factors from granules

NK   Target

$[Ca^{2+}]$

4. Insertion of cytotoxic factors into target cell

5. Lysis of the target cell
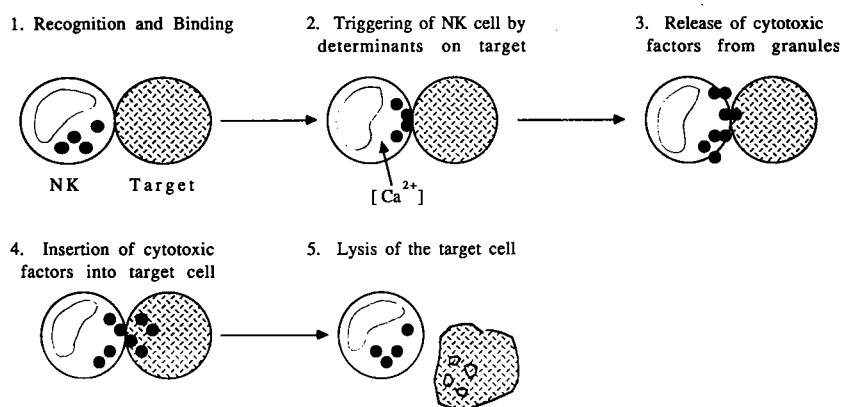
Figure 1.   Steps in the killing of targets by natural killer (NK) cells

NK cells can also bind to and specifically kill target cells that have been sensitized with a specific antiserum or antibody by a phenomenon known as antibody-dependent cell-mediated cytotoxicity (ADCC; van Boxel et al., 1972). In this case, the F_c portion of the IgG antibodies bound to the target cell interacts with the $F_c\gamma$ receptor molecule (CD16) present on the NK cells (Herberman et al., 1977a). The events subsequent to this step in the killing of a target cell by NK cells is the same as conventional NK cell-mediated lysis described below.

2. <u>Triggering of the effector cells</u>: Phosphoinositide turnover is an important component of calcium-dependent cell signalling. Initially, phospholipase C cleaves phosphatidylinositol-4,5-bisphosphate, which generates inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates the release of intracellular calcium ($[Ca^{2+}]_i$) from internal stores, ultimately followed by a $[Ca^{2+}]$ influx from the external environment, whereas DAG activates protein kinase C (PKC), which phosphorylates other proteins required in signal transduction (Nishizuka, 1988; Berridge and Irvine, 1989). This pathway is also important during signalling/triggering events in NK cells. The significance of the role of $[Ca^{2+}]$ in NK cell-mediated lysis is apparent by the inhibition of target cell lysis by EDTA (Welsh, 1978; Hiserodt et al., 1982) or EGTA (Hiserodt et al., 1982; Quan et al., 1982). Upon the interaction of an NK cell with a susceptible target, inositol phosphates (most importantly, IP_3) are rapidly (within 1 min) generated. This has been shown with rat (Gerrard et al., 1987;

Seaman et al., 1987; Atkinson et al., 1989) and human (Chow et al., 1988; Steele and Brahmi, 1988; Windebank et al., 1988; Ståhls and Carpén, 1989; Chow and Jondal, 1990) NK cells. This response is not observed with NK-resistant targets and is inhibited by compounds which prevent phosphoinositide breakdown (Chow and Jondal, 1990). An essential role for PKC in NK cell-mediated lysis has also been demonstrated (Graves et al., 1986; Ito et al., 1988; Chow et al., 1988; Leibson et al., 1990; Hager et al., 1990; Chow and Jondal, 1990), with PKC inhibitors able to block the lysis of the targets in a dose-dependent manner (Ito et al., 1988; Chow and Jondal, 1990; Hager et al., 1990). Following this signalling event, there is a reorientation of the microtubule organizing center and cytoskeleton of the NK cell toward the direction of the target cell (Carpén et al., 1983; Kupfer et al., 1983). This allows granules in the effector cell cytoplasm, which contain cytotoxic moieties, to be positioned at the interface between the effector and target cell.

3. <u>Release of cytotoxic factors from the granules</u>: Early studies of the mechanism of NK cell-mediated cytotoxicity suggested that lysis of the target cell required a secretory event in the effector cell, as compounds such as monensin or others capable of preventing classical secretory mechanisms could inhibit the lytic event (Ortaldo et al., 1980; Carpén et al., 1981). It appeared by electron microscopic analysis that cytotoxic effector cells contained secretory granules, which were surmised to contain factors capable of mediating the lysis of the target cell. Purified granules from CTL or NK/LGL tumor

lines were found to be able to induce the lysis of numerous target cells, much like the effector cells themselves, supporting this hypothesis (Henkart et al., 1984; Podack and Konigsberg, 1984). The electron microscopic studies and biochemical characterization of these granules demonstrated the presence of perforin (Henkart et al., 1984; Podack and Konigsberg, 1984; Young et al., 1986a), which is homologous to complement (C') component C9 (Tschopp et al., 1986; Young et al., 1986b; Zalman et al., 1986), a family of serine esterases (Masson and Tschopp, 1987), in addition to a polyadenylate-binding protein, TIA-1 (Tian et al., 1991), among other factors, which can or may play a role in NK cell-mediated lysis following their release.

4. <u>Death of the target cell</u>: As mentioned above, one component of the granules is the C9 homologue, perforin. In the presence of $[Ca^{2+}]$, polyperforin polymerizes and forms in the membrane of the target cells a channel which, under electron microscopy, resembles that induced by poly C9 itself (Henkart et al., 1984; Podack and Konigsberg, 1984; Young et al., 1986a). These polyperforin channels appear to induce the depolarization of the resting membrane potential, the influx of water, and the leakage of cytoplasmic contents (Young et al., 1986a). Following this is an induction of target cell DNA fragmentation into nucleosome-sized fragments and a "blebbing" of the target cell nuclear and plasma membranes--a process commonly referred to as "apoptosis" or programmed cell death (Cohen et al., 1992).

Inverse correlation between target cell MHC class I antigen expression and sensitivity to NK cell-mediated lysis. In addition to tumor cells, a number of other cells are susceptible to lysis by NK cells, particularly after activation with IFN or IL-2. For example, nontransformed cells, such as thymocytes (Herberman et al., 1977b; Nunn et al., 1977; Hansson et al., 1979), peritoneal cells (Welsh et al., 1979; Kiessling and Welsh, 1980) or bone marrow cells (Nunn et al., 1977), are also sensitive to NK cell-mediated lysis. Furthermore, some virus-infected cells, which will be discussed in more detail below, also appear to be more susceptible than uninfected cells to lysis by NK cells. As it became apparent that NK cells could kill a variety of target cells, it also became clear that the lysis of the targets was major histocompatibility complex (MHC)-independent (Welsh, 1978). In fact, an inverse correlation between target cell MHC class I antigen expression and sensitivity to NK cell-mediated lysis was found with most targets; this was especially the case (though not exclusively) for targets of hematopoietic origin. The expression of low or no MHC class I molecules appeared to confer sensitivity to NK cell-mediated lysis to the target (Ljunggren and Kärre, 1990). Kärre developed what became known as the "missing self" hypothesis. The hypothesis simply states that target cells which do not express sufficiently high levels of self MHC class I will be killed by NK cells. Although the inverse correlation between MHC class I expression and sensitivity to NK cell-mediated lysis is not absolute, there have been a number of different observations by

Kärre and his colleagues, as well as by others, which support this hypothesis.

The prototypic murine NK target cell is a Moloney leukemia virus-induced leukemia cell line, YAC-1 (Kiessling et al., 1975a). These cells express relatively low levels of H-2 class I antigens on their surface, but upon *in vivo* passage as an ascites tumor in syngeneic strain A mice, these cells become much more resistant to NK cell-mediated lysis and express higher levels of H-2 class I antigens (Piontek et al., 1985). Furthermore, treatment of YAC-1 cells with IFN, which upregulates MHC class I antigens (Lindahl et al., 1976), confers resistance of these targets to lysis by NK cells (Welsh et al., 1981; Piontek et al., 1985). In contrast, treatment of target cells with protein synthesis inhibitors, such as cycloheximide, which have the capacity to block MHC class I synthesis (Reichner et al., 1988), renders such targets more sensitive to lysis by NK cells (Kunkel and Welsh, 1981). Obviously, exposure of target cells to reagents which, in addition to altering MHC class I molecules as described above, may also have other global effects which could modulate target cell sensitivity to NK cell-mediated lysis independent of the MHC. Experiments in both human and mouse systems attempted to directly assess the role of MHC class I molecules in the susceptibility of targets to lysis by NK cells. One group used Daudi cells, a human EBV-transformed B cell line which possesses a defect in the β_2-microglobulin (β_2m) gene and are HLA$^-$, and transfected them with a normal copy of the human β_2m gene.

This rescued HLA expression and increased the resistance of these transfectants to killing by NK cells (Quillet et al., 1988). Subsequently, C1R cells, a human lymphoblastoid line which expresses no HLA-A or HLA-B and is very sensitive to lysis by NK cells, were transfected with individual HLA-A or -B genes (Storkus et al., 1989a, b). With one exception (HLA-A2), the transfectants became much more resistant to NK cell-mediated lysis. Exon shuffling experiments mapped the resistance to the $\alpha 1/\alpha 2$ domains of the transgene-expressed HLA molecules (Storkus et al., 1989b).

In the mouse, YAC-1 cells transfected with the K^b gene are more resistant to NK cell-mediated lysis (Carlow et al., 1990), and β_2m-deficient YAC-1 (Ljunggren et al., 1990) or EL-4 (Glas et al., 1992) cells, which are very sensitive to NK cell-mediated lysis in *in vitro* and *in vivo* models, respectively, become more resistant following transfection with a normal β_2m gene. Furthermore, Con A blasts from β_2m-deficient [β_2m (-/-)] mice, which express very low (almost undetectable) levels of free H-2 class I heavy chain (Koller et al., 1990; Zijlstra et al., 1990), are very sensitive to lysis by NK cells from normal mice, whereas Con A blasts from their heterozygous littermates are resistant (Höglund et al., 1991a; Liao et al., 1991). Very strong evidence for a role of H-2 class I antigens in the NK cell-mediated rejection of hematopoietic tumor cells *in vivo* was elegantly demonstrated by Kärre and his colleagues (Höglund et al., 1991b). In this study, H-2^b tumor cells inoculated into H-2^b mice were not rejected. However, these tumor cells were rejected by NK cells when

inoculated into transgenic H-2^b mice which also expressed H-2D^d (D8 mice). As one would predict, H-2^b tumor cells transfected with the D^d gene were able to grow well in D8 mice. However, H-2^b tumor cells transfected with D^q were not rejected in normal H-2^b mice, but were in D8 mice. These results suggested that the tumor cell H-2 class I molecules were delivering a negative signal to (or preventing the triggering of) the recipient's NK cells, and thus the tumors were able to grow. The relevance of this to the recognition of targets by NK cells will be discussed in more detail below. Therefore, a significant body of evidence supports an important role for MHC class I molecules in the sensitivity of target cells to NK cell-mediated lysis.

Recognition of target cells by NK cells: putative receptor molecules. Because of the inverse correlation between target cell MHC class I antigen expression and susceptibility to NK cell-mediated lysis described above, two models to explain a possible role for class I molecules in NK cell-mediated lysis were developed (Figure 2). In the first, the "target interference" model, access to an NK triggering molecule on the surface of the target cell by an NK receptor is obscured or blocked by MHC class I molecules (Figure 2a). With more class I antigens expressed, the more interference (and less triggering of the NK cell) occurs. The end result is a "resistant" target cell. In the second model, the "effector inhibition" model, NK cells have a receptor on their surface which directly interacts with MHC class I molecules (Figure 2b). Upon this interaction, a negative signal
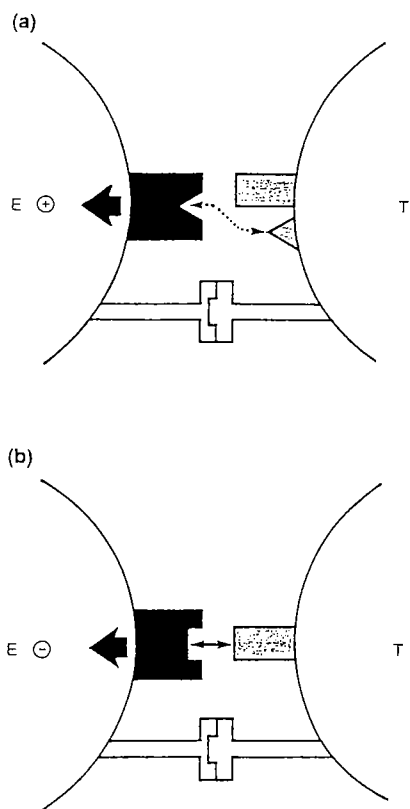
Figure 2. Two models for the role of MHC class I molecules in the sensitivity of target cells to NK cell-mediated lysis. **a)** "target interference" model; **b)** "effector inhibition" model. From Ljunggren and Kärre, 1990.

is delivered to the NK cell, rendering it unable to kill the target. Again, the level of class I on the target would confer either sensitivity or resistance to NK cell-mediated lysis. Recent evidence in both the mouse and human favor the second model.

I mentioned the experiment by Kärre and his colleagues (Höglund et al., 1991b), which presented *in vivo* evidence of a negative signal being delivered to the NK cells. But this is only <u>consistent</u> with this hypothesis. Recently, it has been shown that when H-2^b NK cells (NK1.1$^+$ LAK) are separated on the basis of Ly-49 (found on approximately 20% of NK1.1$^+$ NK cells) (Yokoyama et al., 1990), these NK cells cannot kill targets which express H-2D^d (Karlhofer et al., 1992) or H-2D^k (W. Yokoyama and W. Seaman, personal communication). In contrast, Ly-49$^-$ NK cells could kill all of the H-2^d and H-2^k targets which were resistant to lysis by Ly-49$^+$ NK cells. Furthermore, mAb against either Ly-49 or D^d were able to overcome this block in the ability of Ly-49$^+$ NK cells to kill the H-2D^d-expressing targets (Karlhofer et al., 1992). This suggested that the interaction of Ly-49 and H-2D^d resulted in the delivery of a negative signal to the Ly-49$^+$ NK cells. Comparable evidence for a similar phenomenon in a human system involves the HLA-Cw3 molecule. Human cells or mouse P815 transfectants expressing HLA-Cw3 were resistant to a specific NK cell clone (Ciccone et al., 1992). Anti-HLA-Cw3 antibodies could prevent this block in target cell lysis. As yet, no molecule on the human NK cell clone has been shown to directly interact with HLA-Cw3.

The mAb 3.2.3, originally described as an antibody specific for rat NK cells, identifies a 60-kDa disulfide-linked homodimer nearly exclusively expressed on NK cells (Chambers et al., 1989). The gene encoding the antigen recognized by 3.2.3 has been cloned, revealing the 3.2.3 antigen to be a type II integral membrane protein (C-terminus is on the outside of the cell; Giorda et al., 1990). The moiety recognized by the 3.2.3 mAb has been designated NKR-P1 (NK receptor protein-1). The 3.2.3 mAb can mediate redirected lysis (i.e., NK cells can lyse target cells which have the 3.2.3 mAb bound to their surface via the Fc receptor) (Chambers et al., 1989), stimulate phosphoinositide turnover, and increase $[Ca^{2+}]_i$ in rat NK cells (Ryan et al., 1991)--all characteristics one would predict for a putative NK cell receptor molecule. Subsequently, this gene was cloned in the mouse and found to be one of a family of at least 3 different genes (Giorda and Trucco, 1991; Yokoyama et al., 1991). In fact, one of these 3 NKR-P1 genes has been identified as encoding the mouse NK marker, NK1.1 (Ryan et al., 1992).

As described above, Ly-49 can be used to separate subpopulations of NK1.1+ NK cells (Yokoyama et al., 1990). This molecule has also been cloned (Yokoyama et al., 1989) and NKR-P1, NK1.1, and Ly-49 share many common features: 1) along with the *Cmv-1* gene (murine cytomegalovirus resistance gene, not yet cloned) locus (Scalzo et al., 1992), these genes map to a distal portion of mouse chromosome 6 in a region now referred to as the "NK Complex" (Yokoyama et al., 1991); 2) the proteins are all type II

integral membrane proteins; 3) they share extensive homology with calcium-dependent lectin-binding proteins such as the asialoglycoprotein receptor and the low affinity IgE Fcε receptor (Yokoyama et al., 1989; Giorda et al., 1990; Giorda and Trucco, 1991; Yokoyama et al., 1991). Interestingly, a family of human genes, NKG2, which are restricted to NK cells (Houchins et al., 1991), has also been described. Like the murine genes, they also encode type II integral membrane proteins with putative lectin-binding domains and map to the human homologue of mouse chromosome 6 (Hofer et al., 1992). However, they only share very limited amino acid homology with Ly-49 or NKR-P1 (Houchins et al., 1991). Two different mAb, EB6 and GL183, which have been used to delineate subpopulations of human NK cell clones, immunoprecipitate molecules with a similar molecular weight as the NKR-P1 proteins, although they do not appear to be disulfide-linked (Moretta et al., 1990).

Another molecule in the mouse which may serve as an NK cell receptor is identified by the mAb 5E6. NK cells expressing the 5E6 antigen in an irradiated (C3H x B6)F_1 mouse are able to reject bone marrow grafts from an H-2^d (BALB/c) donor (Sentman et al., 1989), by a phenomenon known as "hybrid resistance" and the target structure(s) or regulatory molecule for the target structure involved has been mapped between the S and D regions of the mouse H-2 complex (Yu et al., 1992). This might suggest that hybrid resistance is an MHC class I-independent phenomenon. Recently, the gene

encoding the 5E6 antigen has been cloned. It is also a class II integral membrane protein, but interestingly, does not map to mouse chromosome 6 (V. Kumar, personal communication).

Another NK cell receptor molecule candidate has been identified using an anti-idiotype antiserum against a human LGL structure, and $F(ab')_2$ fragments of this antiserum can block the lysis of K562 target cells by human NK cells (Frey et al., 1991). Human and mouse cDNA corresponding to this structure have been isolated and shown to be related to the cyclophilins (Anderson et al., 1993). Therefore, NK cells may use a variety of putative receptor molecules in the killing of targets, whether it be by a standard cell-to-cell interaction or by ADCC as mentioned earlier.

Signal transduction molecules present on NK cells. A number of signal transduction molecules, which may or may not play a role in NK cell function, are expressed on the surface of NK cells (some are on other cells as well). Because these molecules are involved in signal transduction and, as I mentioned above, the triggering of an NK cell is an important step in the killing process, I briefly summarize the most important molecules and their involvement (if any) in NK cell activity.

1. Thy 1: A phosphatidylinositol-linked glycoprotein most notably used as a marker for mouse T lymphocytes, as mentioned above, a low density of Thy 1 is also present on NK cells (Herberman et al., 1978). Some evidence has indicated that Thy 1 can act as a

signal transduction molecule in T cells and in Thy 1-transfectants (Kroczek et al., 1986), but its role in the function of NK cells is unknown. A ligand for Thy 1 has yet to be described.

2. <u>CD2</u>: Also known as the sheep erythrocyte receptor, CD2 is a member of the immunoglobulin superfamily of adhesion receptors, as is its ligand, LFA-3 (CD58; Springer, 1990). CD2 appears to play an important role in various human T cell (Bierer et al., 1989) and NK cell (Ritz et al., 1988) responses, but seems to be less important in mouse NK cell activity (Nakamura et al., 1990).

3. <u>CD8</u>: Predominantly a T cell marker for MHC class I-restricted T cells, CD8 is also present on some human (Ortaldo et al., 1981) and all rat (Chambers et al., 1989) NK cells. All mouse NK cells are CD8$^-$. A role for CD8 in T cell signalling is inferred from its association with the *src* family tyrosine kinase, p56^{lck} (Janeway, 1992). At present, CD8 has not been implicated in the function of NK cells.

4. <u>CD16</u>: Also known as the FcγRIII receptor, CD16 is the NK receptor involved in ADCC, as mentioned above. CD16 has been shown to be associated with the ζ chain of CD3, which has been demonstrated to play a major role in T cell signalling via the T cell receptor (Anderson et al., 1989, 1990; Lanier et al., 1989b), as well as the γ-subunit of the high affinity Fc receptor for IgE (FcϵRI; Lanier et al., 1991b; Vivier et al., 1991a). The CD3ζ chain association with CD16 has been demonstrated to be responsible for the induction of the effector phase of ADCC (Vivier et al., 1991b).

5. <u>CD45</u>: A protein tyrosine phosphatase, CD45 is present on all leukocytes (Trowbridge, 1991). The alternative splicing of at least 3 exons (A, B, and C in the mouse) allows for the expression of multiple isoforms of CD45 on the surface of leukocytes and, of particular interest here, lymphocytes (Johnson et al., 1989; Trowbridge, 1991). Although not specifically an NK cell marker, some anti-CD45 antibodies can inhibit NK cell-mediated lysis (Seaman et al., 1981; Newman et al., 1983), in addition to T lymphocyte responses to anti-CD3 and/or IL-2 (Gilliland et al., 1990).

6. <u>CZ-1 antigen</u>: A novel rat mAb generated in this laboratory against purified mouse LGL, mAb CZ-1 stains B cells, CD8$^+$ T cells and NK cells, in addition to activated (but not resting) CD4$^+$ T cells (Vargas-Cortes et al., 1992). mAb CZ-1 also marks the earliest progenitor of B, T and NK cells (Vargas-Cortes et al., 1991). The staining pattern of mAb CZ-1 is unlike any mAb we have seen, but has some similarities (though not identity) with certain anti-CD45 mAb (e.g., inhibition of IL-2-induced responses, Vargas-Cortes et al., 1991), suggesting some role in signal transduction. Therefore, the identification of the moiety recognized by this novel mAb is of interest and is the basis for Chapter VII.

B. Overview of the Immune Response to Virus Infections

Induction of NK cell activity by viruses. Early during the course of a virus infection is the induction of IFNα/β production. As mentioned above, IFN and IFN inducers can activate NK cells by augmenting their lytic capacity (Gidlund et al., 1978; Welsh et al., 1978; Djeu et al., 1979). The appearance of NK cell activity in the spleens of virus-infected mice closely parallels the kinetics of IFNα/β production (Welsh, 1978), and IFN pretreatment of NK cells *in vitro* (Trinchieri and Santoli, 1978; Djeu et al., 1979) or the administration of IFNs *in vivo* (Gidlund et al., 1978; Welsh, 1978; Djeu et al., 1979; Biron et al., 1984) can augment NK cell-mediated target cell lysis. This activation of NK cells begins to wane due, at least in part, to the decline in virus-induced IFN production, as well as the synthesis and release of transforming growth factor-β1 (Su et al., 1991).

Induction of virus-specific cytotoxic T lymphocytes (CTL) and recognition of viral antigens by CTL. As the NK cell response decreases over time, the generation of virus-specific CTL occurs and peaks approximately 7 - 9 days post-infection with lymphocytic choriomenigitis virus (LCMV; Welsh, 1978). Concomitant with the induction of virus-specific CTL, virus-induced allospecific CTL activity, although at a level below that observed for virus-specific CTL, can be detected in both mice (Yang and Welsh, 1986) and humans (Tomkinson et al., 1989).

Viral proteins are degraded into small fragments in the cytoplasm of infected cells (Townsend and Bodmer, 1989) by processes which may include the participation of the recently described proteosome components, some of which map to regions in the MHC class II gene loci (Brown et al., 1991; Glynne et al., 1991). These peptide-sized fragments are then believed to be transported into the endoplasmic reticulum by a heterodimeric complex encoded by the genes, TAP-1 and TAP-2 (Deverson et al., 1990; Monaco et al., 1990; Spies et al., 1990; Trowsdale et al., 1990). The importance of the TAP-1 and TAP-2 gene products in peptide transport is evident in mutant cells, defective for either the TAP-1 (721.134 cells; Spies and DeMars, 1991) or TAP-2 genes (RMA-S cells; Attaya et al., 1992). In the endoplasmic reticulum or other pre-Golgi compartment, peptides form a heterotrimeric complex with the MHC class I heavy chain and β_2-microglobulin (Yewdell and Bennink, 1992). The association of peptide and MHC class I molecules was identified using 3 different approaches: 1) the exogenous addition of small synthetic peptide fragments (~15 amino acid residues) to appropriately MHC-matched targets resulted in a sensitization of the targets for lysis by MHC class I-restricted, virus-specific polyclonal CTL or CTL clones (Maryanski et al., 1986, 1988; Townsend et al., 1986; Braciale et al., 1987; Gotch et al., 1987; Pala et al., 1988); 2) the crystal structure of the human MHC class I molecule, HLA-A2, showed an electron dense material in the cleft of the HLA-A2 molecule which appeared to be peptide in an extended conformation (Bjorkman et al., 1987); 3)

material extracted from virus-infected cells associated with MHC class I molecules were HPLC-purified, sequenced, and shown to be peptides of 8 or 9 amino acids in length (Rötzschke et al., 1990; Van Bleek and Nathenson, 1990). These peptides corresponded to previously-defined minimal immunodominant epitopes for virus-specific CTL using synthetic peptides of various lengths. Furthermore, it could be shown that preparations of the larger (~15-mer) synthesized peptides, which defined the CTL epitopes, contained the natural peptide sequences as "contaminants". In fact, these "contaminants" could sensitize the appropriate targets for lysis by specific CTL much more efficiently than the larger peptides (Rötzschke et al., 1990). More recent studies analyzing peptides derived from uninfected cells in both mice (Falk et al., 1991) and humans (Falk et al., 1991; Jardetzky et al., 1991) have also found peptides of 8 or 9 amino acids complexed with the MHC class I antigens. In infected cells, as well as uninfected cells, following the charging of an MHC class I molecule with an 8 or 9 amino acid peptide, either in the endoplasmic reticulum or other pre-medial Golgi compartment (del Val et al., 1992), the heterotrimeric complex traverses through the protein secretory pathway, and is expressed on the surface for recognition by virus-specific CTL in an MHC-restricted manner (Figure 3; Townsend and Bodmer, 1989; Yewdell and Bennink, 1992).
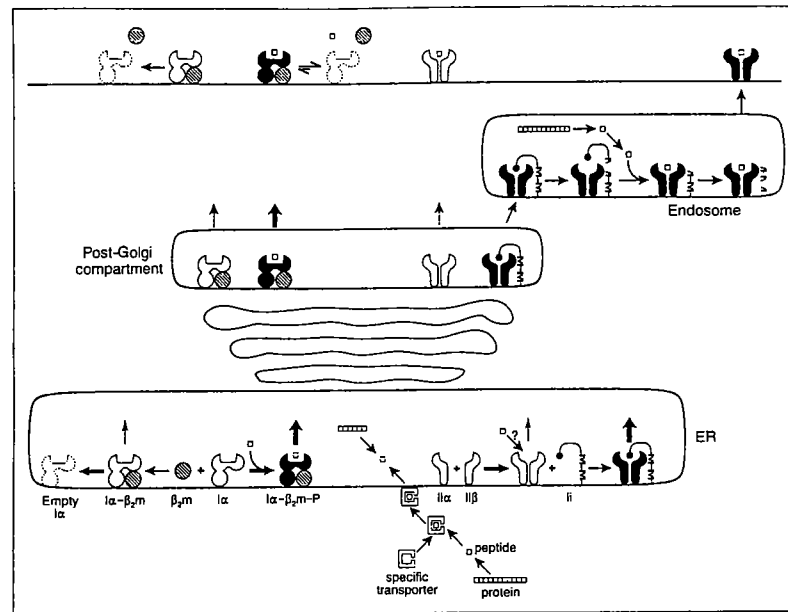
Figure 3. Processing and presentation of peptide antigens on MHC class I or class II molecules. In this thesis, the emphasis is on the processing of endogenous viral peptide antigen on MHC class I molecules (left half of cartoon) for presentation to virus-specific CTL and NK cells. Proteins are degraded into peptide-sized (< 15 amino acid residues) and transported into a pre-Golgi (probably endoplasmic reticulum) compartment by a specific transporter molecule(s), where the peptides charge the MHC class I molecules, traverse through the Golgi complex and subsequently expressed on the surface of the cell. From Braciale, T. J., and V. L. Braciale. 1991. Immunology Today 12:124-129.

C. Sensitivity of Virus-Infected Cells to
NK Cell-Mediated Lysis

Early studies. The first reports analyzing the susceptibility of virus-infected targets to NK cell-mediated lysis suggested that following a virus infection, human target cells were more sensitive to lysis by human NK cells in overnight cytotoxicity assays (Trinchieri and Santoli, 1978). A number of different viruses were used, and most gave the same result. This experimental design did not allow one to distinguish between NK cell activation and the innate susceptibility of the target to lysis by NK cells, as significant levels of IFN_α were produced in those long, overnight assays (Santoli et al., 1978a, b). This IFN, now known to be made predominantly by Ia^+ dendritic-like cells (Bandyopadyay et al., 1986; Fitzgerald-Bocarsly et al., 1988), activates NK cells to higher levels of cytotoxicity, and enables the NK cells to lyse the virus-infected target cells in the vicinity. Experiments designed to bypass an overt NK cell activation phenomenon have used short-term (e.g., 3-5 h) cytotoxicity assays (Welsh and Hallenbeck, 1980), highly activated NK cells (Welsh, 1978; Welsh and Hallenbeck, 1980; Borysiewicz et al., 1985), and/or nonactivated NK cells incubated with antibodies to $IFN\alpha/\beta$ or with metabolic inhibitors which block IFN synthesis or action (Casali and Oldstone, 1982; Fitzgerald et al., 1982; Bishop et al., 1983; Bandyopadhyay et al., 1986). Experiments designed in that manner have shown that cells infected with any of several viruses, such as

herpes simplex virus type 1 (HSV-1; Welsh and Hallenbeck, 1980; Muñoz et al., 1983), murine cytomegalovirus (MCMV; Bukowski and Welsh, 1985), Sendai virus (Welsh and Hallenbeck, 1980) and vaccinia virus (VV; Welsh et al., 1989), can often be rendered more resistant than uninfected targets to NK cell-mediated lysis.

Role of increased NK cell binding to virus-infected targets or enhanced triggering of the effector cells. Work with some viruses, such as vesicular stomatitis virus (VSV), has correlated enhanced binding to enhanced killing (Moller et al., 1985), but in other systems, such as Sendai virus, enhanced binding can occur without enhanced lysis (Welsh and Hallenbeck, 1980). Given that NK cells can bind to most target cells in the absence of virus infection, it would seem that a post-binding event might be of greater significance in enhancing the lysis of virus-infected targets. Cold target competition assays have often shown that virus-infected cells compete better than uninfected cells against NK cell-mediated lysis of target cells (Minato et al., 1979; Blazar et al., 1980; Welsh and Hallenbeck, 1980); this has often been interpreted as an indicator of enhanced binding of NK cells to the virus-infected targets, but it also could be interpreted to mean enhanced triggering of the NK cells and secretion of their cytotoxic moieties. Of note is that IFN can protect target cells from NK cell-mediated lysis by inhibiting triggering without inhibiting binding, and IFN-treated target cells fail to compete in cold target competition assays (Trinchieri and Santoli,

1978; Welsh and Hallenbeck, 1980; Trinchieri et al., 1981; Welsh et al., 1981).

Role of viral glycoproteins in enhancing target cell lysis by NK cells. Another aspect of a virus infection which might play a role in the sensitivity of virus-infected targets to NK cell-mediated lysis is the expression of viral glycoproteins. Soon after infection of cells with a number of viruses, serologically detectable viral glycoproteins are present on the surface. Glycoproteins purified from influenza (Arora et al., 1984), LCMV and measles (Casali et al., 1981), mumps (Härfast et al., 1980), and Sendai (Alsheikhly et al., 1983) viruses increase target cell lysis by NK cells. The NK cell-mediated killing of HSV-1-infected fibroblasts has been reported to be inhibited by F_{ab} fragments obtained from the serum of HSV-1 seropositive donors or by mAb specific for the HSV-1 glycoproteins gB and gC (Bishop et al., 1984). In contrast, antisera or mAb specific for VSV glycoproteins do not alter the killing of VSV-infected targets by NK cells, and the cell surface expression of VSV glycoproteins (G) in VSV G-transfected COS cells does not render them more sensitive to lysis by NK cells (Moller et al., 1985). However, targets infected with temperature-sensitive VSV glycoprotein mutants and grown at the nonpermissive temperature are not killed very well, whereas those grown at the permissive temperature are very susceptible to NK cell-mediated lysis (Moller et al., 1985). It is unclear at what step of the killing process these glycoproteins might act, but the augmented killing

observed in most of these systems appears to be IFN-independent, regardless of whether soluble viral glycoproteins were directly added to effector cells and then washed out (Casali et al., 1981), or if virus-infected targets were used (Bishop et al., 1983; Moller et al., 1985). Although the expression of viral glycoproteins may be associated with an increase in binding of effectors to targets (Welsh and Hallenbeck, 1980; Moller et al., 1985), this does not always result in an increase in target cell lysis. Therefore, a role for viral glycoproteins in conferring enhanced susceptibility of target cells to NK cell-mediated lysis may depend upon the virus.

Role of MHC class I antigens in the sensitivity of virus-infected target cells to NK cell-mediated lysis. As mentioned above, there is an inverse correlation between target cell MHC class I antigen expression and susceptibility to lysis by NK cells. One would predict, therefore, that those viruses which decrease the levels of MHC class I molecules in a particular target cell would make that cell more sensitive to NK cell-mediated lysis. A number of different groups of viruses do reduce MHC class I expression (Maudsley and Pound, 1991). Of these, the most highly cytopathic viruses will inhibit host cell protein synthesis, with a resultant decrease in MHC class I expression. Other viruses reduce MHC class I antigens without adversely affecting other cellular proteins. For example, the E1A product of adenovirus 12 inhibits MHC class I gene expression at the level of transcription (Friedman and Ricciardi, 1988), whereas the

19-kDa product (E19) of the E3 gene of several adenovirus serotypes specifically binds to the heavy chain of the class I molecule and retains it in the endoplasmic reticulum (Burgert et al., 1987; Tanaka and Tevethia, 1988; Cox et al., 1990, 1991). Some viruses, such as human immunodeficiency virus-1 (HIV-1; Scheppler et al., 1989), inhibit MHC class I expression by unknown mechanisms. Other than the work to be presented in this thesis, in only one report (Dawson et al., 1989), in which adenovirus-infected cells were tested, has virus-induced alterations in MHC class I antigen expression been correlated with sensitivity to lysis by NK cells, although other studies in the adenovirus system have not seen such a correlation (Routes, 1992, 1993).

I had mentioned above that a number of HLA class I molecules transfected into the HLA-A$^-$/HLA-B$^-$ C1R cells could confer NK-resistance to such transfectants, with the exception of HLA-A2, and that this property mapped to the $\alpha 1/\alpha 2$ domains (Storkus et al., 1989a, b). These same investigators then showed that a single substitution of the His-74 residue to an Asp in the HLA-A2 molecule was sufficient to allow this molecule to confer NK-resistance upon transfection into C1R cells (Storkus et al., 1991). Interestingly, His-74 is near the peptide-binding groove in HLA-A2 and interferes with access to a side pocket in the groove (Bjorkman et al., 1987). This suggested that the peptides residing in the groove may be an important factor in whether or not a particular target cell is NK-sensitive or NK-resistant. Therefore, alterations in the peptides

presented would be predicted to modulate sensitivity to NK cell-mediated lysis. This question will be addressed in detail in my thesis.

OBJECTIVE OF THESIS

The overall objective of this thesis has been to study the regulation of a virus infection by NK cells. The major focus was on cell surface molecules present on the target or effector cells which could modulate the triggering of NK cells, and could therefore play a role in *in vitro* and *in vivo* events mediated by NK cells.

The molecules to be addressed here, which are present on the target cells and can negatively influence signal transduction in NK cells, are H-2 class I molecules, whereas the molecule on the surface of NK cells which also appears to have an effect on NK cell triggering is identified by the mAb CZ-1. The following specific questions were asked:

1. Is there is an enhanced sensitivity of virus-infected target cells to NK cell-mediated lysis during a discrete period of time post-infection?

2. If so, at what stage of the lytic process does this occur and, how does this affect signal transduction in NK cells?

3. Under conditions of a virus infection of target cells which alter sensitivity to NK cell-mediated lysis, is there a change in the susceptibility of these targets to lysis by allospecific or virus-specific CTL?

4. Does viral peptide charging of H-2 class I molecules modulate target cell sensitivity to NK cell-mediated lysis (and therefore NK cell triggering) *in vitro*?

5. Are H-2 class I molecules important in the regulation of a virus infection by NK cells *in vivo*?

6. Are H-2 class I molecules required in IFN-mediated protection from NK cell activity *in vitro* and/or *in vivo*?

7. What is the identity of the signal transduction molecule on NK (and other cytotoxic effector) cells recognized by the mAb CZ-1?

CHAPTER II

MATERIALS AND METHODS

A. Animals

Male A/J (H-2^a), BALB/cByJ (H-2^d), C3H/HeSnJ (H-2^k), C57BL/6 (H-2^b) and C3H.OH (H-2K^d, -D^k) mice were purchased from The Jackson Laboratory, Bar Harbor, ME and were used at 6 - 8 weeks of age. Mice harboring the severe combined immunodeficiency (SCID) mutation (CB.17, H-2^d background) were derived from breeders originally provided by Dr. Donald Mosier, Medical Biology Institute, La Jolla, CA) and were bred and maintained under germfree conditions in facilities at the University of Massachusetts Medical Center (UMMC). The SCID mice were used at 4 - 6 weeks of age. Breeder pairs of mice homozygous for a targeted mutation disrupting the gene for β_2-microglobulin [β_2m (-/-)], originally derived by Drs. B. Koller and O. Smithies (Koller et al., 1990), University of North Carolina, Chapel Hill, NC, were kindly supplied by Dr. D. Roopenian (The Jackson Laboratory). These mice were housed under specific pathogen-free conditions and were used at 6 - 12 weeks of age.

B. Cell Lines

YAC-1 cells are a Moloney virus-induced leukemia from A/Sn mice; P52 is a T cell lymphoma from H-2^b mice; P815 cells are a mastocytoma from DBA/2 (H-2^d) mice; R1.1 cells are a thymoma derived from C58/J (H-2^k) mice; R1E cells are derived from R1.1 cells and are defective in β_2m gene expression (Allen et al., 1986); D1R

(Waneck et al., 1987) and R1E + D^b (Allen et al., 1986) are R1.1 and R1E cells transfected with the H-2D^b gene, respectively; R1.1, R1E, D1R and R1E + D^b were provided by Dr. G. Waneck, Massachusetts General Hospital; 18.81 cells (H-2^d) are a pre-B cell line provided by Dr. J. Stavnezer, UMMC. YAC-1, P52, P815, R1.1 and R1E were propagated in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2mM L-glutamine and antibiotics (complete medium). D1R and R1E + D^b were grown in complete medium further supplemented with 500 μg/ml of G418 (Gibco/BRL, Gaithersburg, MD). 18.81 cells were propagated in complete medium with 10 mM HEPES buffer, 5×10^{-5} M 2-mercaptoethanol, 0.1 μM sodium pyruvate and 0.1 mM nonessential amino acids as further supplements (MLC-medium). L929 cells, a fibroblast line from C3H (H-2^k) mice, the low H-2 class I-expressing L929 variants, 5a-15:9 and 4a-1:3, in addition to L929 cells persistently-infected with LCMV [L(LCMV)], were grown in Eagle's minimum essential medium (MEM, Gibco) with the same supplements as for complete medium. DAP-3 cells, an Ltk$^-$ L cell derivative and DAP-3 cells transfected with the genes for H-2D^b (L-D^b) and H-2L^d (L-L^d) (Lee et al., 1988) were propagated in Dulbecco's MEM, with complete medium supplements, and were obtained from Dr. T. Hansen, Washington University, St. Louis, MO. Mouse embryo fibroblasts (MEF) from C57BL/6 mice were maintained in Eagle's MEM as above. Ψ2 parental and Ψ2 transfectant cell lines expressing the CD45 alternatively spliced exons A, B and C (T200/ABC), exons B

and C (T200/BC), exon C (T200/C), or none of the alternatively spliced exons (T200/0), were obtained from Dr. I. Trowbridge, Salk Institute, San Diego, CA. These cells were grown in Dulbecco's MEM with 8% FBS, L-glutamine and antibiotics.

C. Viruses

The Armstrong strain of LCMV was prepared in BHK cells (Welsh, 1978). The WR strain of vaccinia virus (VV) was propagated in L929 cells (Bukowski et al., 1983). A recombinant VV expressing a truncated form of the HIV-1 (IIIB) envelope gene (VV-abtΔ74-79) was provided by Dr. J. Sullivan, UMMC. Mouse hepatitis virus (MHV) strain A-59 was plaque-purified in L929 cells and grown to a titer of 7×10^6 plaque-forming units (PFU)/ml. The Smith strain of MCMV was obtained from the salivary glands of C57BL/6 or BALB/c mice, 3 weeks following i.p. infection. The Indiana strain of VSV was cultured in L929 cells. Mice received i.p. injections of all viruses with the exception of VSV, which was administered via the retroorbital sinus (i.o.).

D. Antibodies and Reagents

The 16-3-1N and 15-5-5S hybridomas secrete monoclonal allotypic anti-H-2K^k and anti-H-2D^k antibodies, respectively. The 28-11-5S and 28-14-8S hybridomas produce anti-H-2D^b alloantibodies that recognize conformational-dependent or -independent determinants, respectively (Townsend et al., 1989). The rat

anti-mouse H-2 class I mAb (clone M1/42.3.9.8.HLK) is panreactive for all mouse H-2 class I molecules (except H-2D^b). These five hybridomas were obtained from the American Type Culture Collection, Rockville, MD. CZ-1 is a rat IgM (κ), generated in this laboratory by immunizing BB/W rats with Percoll-enriched activated mouse large granular lymphocytes (Vargas-Cortes et al., 1992). J11d produces a monoclonal rat antibody which recognizes a determinant on murine B cells and neutrophils (Bruce et al., 1981). Monoclonal anti-Lyt 2 antibody was provided as an ammonium sulfate-cut supernatant by Dr. E. Martz, Univ. of Massachusetts/Amherst. The following anti-CD45 restricted, exon-specific mAb were used: the exon A-specific 14.8 (Kincade et al., 1981) and the exon C-specific DNL 1.9 (Dessner and Loken, 1981) mAb were provided by Dr. David Parker, UMMC; the B exon-specific CD45R-1 (clone 16A; Bottomly et al., 1989), unlabelled, biotinylated, or conjugated to RED613 (Gibco/BRL); and the pan-CD45-reactive clone M1/9.3.HL.2 (Springer et al., 1978), unlabelled, biotinylated, or directly conjugated with fluorescein isothiocyanate (FITC; Gibco/BRL). Most monoclonal antibodies used in FACS analyses were used as crude supernatants as described below, with the exception of CZ-1 and the anti-CD45 mAb (other than those that were purchased), which were ammonium sulfate-concentrated supernatants, and the phycoerythrin (PE)-labelled anti-CD4 and FITC-labelled anti-CD8 mAb, which were purchased from Gibco/BRL and used neat. PE- or FITC-labelled goat anti-rat IgM antiserum (μ-chain-specific) was obtained from

Southern Biotechnology Associates (Birmingham, AL). FITC-conjugated goat anti-rat IgG (γ-chain-specific) antiserum was also purchased from Southern Biotechnology Associates. Phycoerythrin-conjugated streptavidin (SA-PE) was obtained from Becton Dickinson (Mountain View, CA). Rabbit antiserum against asialo GM_1 was purchased from Wako (Dallas, TX). Purified mouse natural $IFN\beta$ (800,000 Units) was obtained from Lee Biomolecular (San Diego, CA). Recombinant human IL-2 was obtained from Cellular Products, Inc. (Buffalo, NY). Cycloheximide, sialic acid, and N-acetylneuramin-lactose were all purchased from Sigma.

E.   Interferon-β ($IFN\beta$) and Cycloheximide Treatment of Targets

YAC-1, R1.1, R1E, D1R and R1E + D^b cells were treated with 1000 U/ml of $IFN\beta$ for 18-24 hr at 37°C. L929 cells were incubated with 25 μg/ml of cycloheximide for 18-20 hr, as previously described (Kunkel and Welsh, 1981). The treated cells were washed twice in the appropriate medium before being added to the assays as described below.

F.   Effector Cell Generation

Activated NK cells. Mice were infected i.p. with 5 x 10^4 PFU of LCMV. Alternatively, poly I:C (100 μg/mouse, i.p.) was administered to the mice. Two (poly I:C) or 3 days (LCMV) later, spleens were harvested, processed and introduced into the cytotoxicity assays at various effector to target cell (E:T) ratios.

Activated peritoneal exudate (PEC) NK cells. C57BL/6 mice were infected i.p. with 7 x 10^5 PFU of MHV. On day 3 post-infection, the mice were sacrificed and the PEC were obtained. Macrophages were removed by adherence on FBS-coated petri dishes. The nonadherent cells were removed, washed twice in complete medium, and treated with J11d followed by rabbit complement (C'; Pel-Freeze Biologicals, Brown Deer, WI). Dead cells were removed by density gradient centrifugation on Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada). The cell number was then adjusted as required and used in [$^{45}Ca^{2+}$] influx assays as described below.

Virus-specific CTL. Mice received i.p. injections of 1 - 10 x 10^6 PFU of VV, 5 x 10^6 PFU of VV-abtΔ 74-79, 5 x 10^4 PFU of LCMV, or an i.o. infection with 1 x 10^6 PFU of VSV. On days 0 and 4 post-infection, the mice also received i.p. injections of 10 μl (BALB/c and C57BL/6) or 20 μl (C3H/HeSnJ) anti-asialo GM_1 antiserum. Six to 9 days later, single cell suspensions of spleen cells were prepared and used as effectors in ^{51}Cr release assays as described below.

In vivo-generated allospecific CTL (Figure 4). A/J (H-2K^k, -D^d), C3H/HeSnJ (H-2^k), or C3H.OH (H-2K^d, -D^k) mice were irradiated with 750 R of γ-irradiation (^{137}Cs source, Gammacell 40, Atomic Energy of Canada Ltd., Ottawa, Canada). One hour later, the mice received 5 - 10 x 10^7 spleen cells from either BALB/c (H-2^d) or C57BL/6 (H-2^b) mice by i.o. injection. Five days after transfer, spleen cells from the recipient mice were prepared and used in the cytotoxicity assays.

IN VIVO GENERATION OF
ALLOSPECIFIC CTL

750 R

C3H/HeSnJ (H-2K^{k}D^k)
or
A/J (H-2K^{k}D^d)
or
C3H.OH (H-2K^{d}D^k)

1 hour

inject 5 - 10 x 10^7 BALB/c (H-2^d) or
C57BL/6 (H-2^b) spleen cells, i.o.

5 days

isolate recipient splenocytes

^{51}Cr release assay
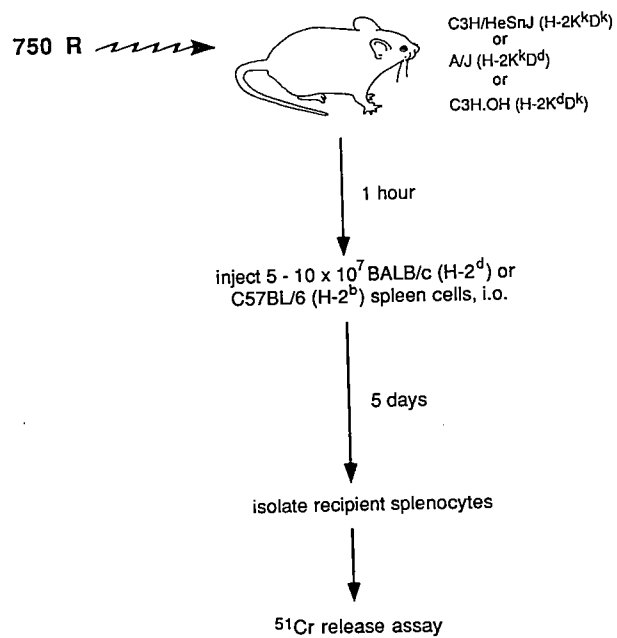
Figure 4. *In vivo* generation of allospecific cytotoxic T lymphocytes
(CTL).

<u>Lymphokine-activated killer (LAK) cells.</u> Spleens from CB.17 SCID or normal C57BL/6 mice were aseptically removed, and single cell suspensions were prepared and adjusted to a final cell concentration of 3×10^6 cells/ml. Recombinant human IL-2 was added for a final concentration of 1000 U/ml. After 5 days at 37°C, the cells were washed twice in complete medium and were used as effectors at various E:T ratios in the ^{51}Cr release assays.

G. Target-Binding Cell Assay

Spleens from C3H/HeSnJ mice were harvested 3 days after LCMV infection, and a single cell suspension was prepared by homogenizing the spleens between the frosted ends of two microscope slides. The spleen cell suspension was then passed over a nylon wool column and mixed with uninfected or VV-infected L929 cells at an E:T ratio of 5:1. The cells were then pelleted (300 x g) for 5 min and placed at 4°C for 30 min. The pellets were gently resuspended and effector/target cell conjugates were enumerated by microscopic examination in a hemacytometer.

H. ^{51}Cr Release Assay

Standard 4 - 6 h ^{51}Cr release assays were performed as previously described (Yang and Welsh, 1986). Briefly, 1×10^6 target cells were incubated with 100 µCi of $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL) for 60-90 min at 37°C. The targets were washed three times in complete medium, and 1×10^4 or 4×10^4

(thymocytes only) cells (in 100 μl) were added to the appropriated wells of flat or U bottom 96 well tissue culture plates (Costar, Cambridge, MA). An equal volume of effector cells was then added per well at various E:T ratios.

Modifications of this assay for different types of experiments are described as follows:

<u>VV-infected targets.</u> L929 cells were preseeded into 6-well tissue culture plates (5×10^5), 60-mm tissue culture dishes (1×10^6) or 25-cm^2 tissue culture flasks (1×10^6 - 2×10^6; Costar). After allowing for adherence (≥ 4 h), the cells were infected with VV-WR at a multiplicity of infection (MOI) of 10 for various intervals at 37°C. The MOI was based on the initial cell input. The cells were dispersed with trypsin, washed twice with complete medium and labelled with ^{51}Cr and used as targets as described above. In some experiments, uninfected L929 cells were prelabelled with ^{51}Cr, plated at 1×10^4 cells/well in flat bottom 96-well plates, and infected with VV (MOI=10) for various time periods before effector cells were added for the ^{51}Cr release assays.

<u>Peptide-treated targets.</u> Two different methods of treating target cells with peptides were used: a) 1×10^6 target cells were treated with or without 200 - 250 μM of the indicated synthetic peptides and 100 μCi of ^{51}Cr in 1.0 ml complete medium, incubated overnight (16 - 18 h) at 37°C, and washed 3 times in complete medium before being added to the assay; b) target cells were prelabelled with 100 μCi of ^{51}Cr for 1 h as described above and

plated (1 x 10^4 cells/well in 50 μl) in 96 well round or flat bottom plates (Costar); peptides were added for a final concentration of 10 μg/ml, and the plates were preincubated for 30 min at 37°C before the addition of effector cells at various E:T ratios.

I. Peptides

The peptides used were the following (see also Table 1): K^b-restricted HSV-1 $gB_{497-507}$ (TSSIEFARLQF), D^b-restricted influenza $NP_{365-379}$ (IASNENMETMESSTL), D^d-restricted HIV-1-IIIB $gp160_{315-329}$ (RIQRGPGRAFVTIGK), K^k-restricted influenza NP_{50-62} (SDYEGRLIQNSLI), K^d-restricted influenza $HA_{147-161}$ (TYQRTRALVRTGMDP) and K^k-restricted rabies $NS_{191-206}$ (EKDDLSVEAEIAHQI) peptides, were purchased from Chiron Mimotopes (Clayton, Victoria, Australia). D^b-restricted LCMV $NP_{397-407}$ (QPQNGQFIHFY), D^b-restricted LCMV $GP_{278-286}$ (VENPGGYCL), L^d-restricted LCMV $NP_{119-127}$ (PQASGVYMG), L^d-restricted MCMV $IEp89_{168-176}$ (YPHFMPTNL), and K^b-restricted VSV N_{53-59} (GYVYQGL), in addition to the D^b-restricted influenza and K^k-restricted rabies peptides were synthesized by Dr. Robert Carraway, UMMC. These peptides were purified to >95% homogeneity by reverse-phase HPLC and peptides were further analyzed by amino acid analysis. The K^d-restricted influenza $HA_{515-526}$ (ILAIYSTVASSL) peptide was kindly provided by Dr. F. Ennis, UMMC. Peptides were dissolved in DMSO or water (as required) at a stock concentration of 1 x 10^5 μg/ml before dilution in complete medium for the assays.

TABLE 1

Synthetic Viral Immunodominant Peptides Used in this Study

Virus (epitope)	Sequence	Presenting H-2 Class I Molecule
HIV-1-IIIB (gp160$_{315-329}$)	RIQRGPGRAFVTIGK	D^d
HSV-1 (gB497-507)	TSSIEFARLQF	K^b
Influenza (HA$_{147-161}$)	TYQRTRALVRTGMDP	K^d
Influenza (HA515-526)	ILAIYSTVASSL	K^d
Influenza (NP50-63)	SDYEGRLIQNSLI	K^k
Influenza (NP365-379)	IASNENMETMESSTL	D^b
LCMV (GP278-286)	VENPGGYCL	D^b
LCMV (NP119-127)	PQASGVYMG	L^d
LCMV (NP397-407)	QPQNGQFIHFY	D^b
MCMV (IEpp89168-176)	YPHFMPTNL	L^d
Rabies (NS191-206)	EKDDLSVEAEIAHQI	K^k
VSV (N53-59)	GYVYQGL	K^b

J. Generation of Con A Blasts

Spleen cells were obtained from the indicated mouse strains and single cell suspensions were aseptically prepared. One x 10^7 spleen cells/ml were treated with 2 μg/ml of concanavalin A (Con A; Sigma) for 48 h at 37°C. The viable cells were obtained at the interface after density gradient centrifugation on a Lympholyte-M gradient. The cells were then washed twice in complete medium, and 1 x 10^6 cells were labelled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 h at 37°C as described above before use as targets in a 4- to 6-h ^{51}Cr release assay.

K. NK Cell Depletion *In Vivo*

NK cells were depleted *in vivo* by i.p. injection of 10 - 20 μg of anti-asialo GM_1 antiserum that was administered at the same time as the virus infections (Habu et al., 1981; Kawase et al., 1982).

L. FACS Analyses

Standard single color indirect immunofluorescent staining technique. For VV-infected L929 cells, the cells were seeded into 25-cm^2 tissue culture flasks and after overnight adherence at 37°C, infected with VV-WR for various time intervals at an MOI of 10. At the end of the infection period, the virus was UV-inactivated by exposure for 15 min to shortwave UV light (254 nm). For L929 (VV-infected and uninfected), 18.81 and the Ψ2 transfectant cells, 200 μl of the appropriate hybridoma supernatant (or 5 μl of the

commercially prepared anti-CD45 mAb) were added to 1 x 10^6 cells. After a 30 min incubation on ice, the cells were washed and labelled with FITC-conjugated goat anti-mouse Igs (IgA + IgG + IgM, H and L chains; Cappel Laboratories, Westchester, PA). The cells were then incubated for 30 - 45 min on ice. After washing, the cells were fixed in 4% paraformaldehyde for 10 min on ice, washed, and kept at 4^oC until FACS analysis by a FACScan IV (Becton Dickinson). For thymocyte expression of H-2D^b, thymi were obtained from uninfected or MCMV-infected (3 days post-infection) C57BL/6 or β_2m (-/-) mice and homogenized into single cell suspensions. The thymocytes were then stained with the conformational-dependent (28-11-5S) or conformational-independent (28-14-8S) anti-H-2D^b monoclonal antibodies (Townsend et al., 1989) from hybridoma supernatants as performed above.

Two color direct immunofluorescence with thymocytes. For detection of thymocyte CD4 and CD8, thymi from individual uninfected or MCMV-infected (3 days post-infection) mice were harvested and single cell suspensions were prepared. The cells were then stained with 5 µl of both PE-labelled anti-CD4 and FITC-conjugated anti-CD8 per 1 x 10^6 thymocytes on ice for 30 min, washed, and fixed as above. Two color analysis was by FACStar (Becton Dickinson).

Competitive FACS analysis. The pre-B cell line, 18.81, was treated with mAb CZ-1 or J11d, in addition to the various anti-CD45 mAb. The cells were then stained with a PE-labelled goat anti-rat

IgM (μ-chain-specific) antiserum, and a FITC-conjugated goat anti-rat IgG (γ-chain-specific) antiserum. For competitive FACS analysis of the Ψ2 transfectants, CZ-1 and either biotinylated anti-CD45RB or anti-CD45 framework antibodies were added together, washed and then labelled with a FITC-labelled goat anti-rat IgM (μ-chain-specific) and SA-PE. Two color analysis was by FACScan (Becton Dickinson).

M. Triggering Assay

A measurement of $[^{45}Ca^{2+}]$ uptake into cocultures of NK and target cells was performed by a modification of that previously described for human NK cells (Jondal et al., 1986; Grönberg et al., 1988). Additionally, this assay was adapted for use in microtiter plates. For the microassay, 5 μCi of $^{45}CaCl_2$ (ICN Biomedicals, Irvine, CA) in assay medium containing 10 mM HEPES buffer (Sigma) was added to each well of a 96-well V-bottomed microtiter plate (Costar). Splenic effector cells from SCID mice 3 days post-LCMV infection (Welsh et al., 1991) were enriched for NK cells by treatments with J11d and C', followed by Lympholyte-M centrifugation. The effector cell concentration was adjusted to 2×10^6 cells/ml and 1×10^5 cells were added per well. L929 cells, obtained at various intervals post-VV infection (MOI = 10), were added to the appropriate wells at an E:T ratio of 1:1. The plates were centrifuged gently (50 x g) for 1 min and incubated for 30 min at 37°C. This time point was chosen in order to maximize the time for NK cell triggering prior to lysis of the

target cell. The reaction was stopped by adding ice-cold 5 mM $NiCl_2$ (Sigma) in assay medium. The plates were then centrifuged at 500 x g for 10 min at 4°C. After 2 more washes in assay medium containing $NiCl_2$, the cells were lysed with 200 μl of distilled H_2O, and the supernatant was added to 2 ml of scintillation fluid (Ecolite; ICN) and counted in a liquid scintillation counter (LKB Instruments; Rockville, MD). The net influx of $[^{45}Ca^{2+}]$ was then determined.

N. Plaque Assay

Spleens from MCMV-infected mice were harvested 3 days post-infection, placed in 1 ml of complete medium on ice and homogenized in a mortar and pestle device. Supernatants were clarified by centrifugation (2000 x g, 15 min at 4°C). Ten-fold serial dilutions of 100 μl were added to the appropriate wells of 6-well tissue culture plates (Costar), which were preseeded 2 days previously with approximately 1×10^5 C57BL/6 mouse embryo fibroblasts (early passage) per well and contained 1 ml complete MEM. After a 90 min absorption at 37°C, the plates were overlayed with 4.0 ml of a 1:1 (v/v) mixture of 2X medium 199 (Gibco/BRL) and supplements and 1% agarose (SeaKem ME, FMC Corp., Rockland, ME). After 5 days at 37°C in a humidified atmosphere, the overlay was removed, and the plates were stained with crystal violet. The virus titers are indicated as plaque forming units (PFU)/spleen.

O.   Statistical Analysis

For the determination of p values, Student's t-test was used.

CHAPTER III

STUDIES ON TARGET CELL SUSCEPTIBILITY TO NK CELL-MEDIATED LYSIS FOLLOWING VV INFECTION

A. "Window of Vulnerability" of Vaccinia Virus (VV)-Infected L929 Cells to NK Cell-Mediated Lysis

As mentioned in Chapter I, early studies found that various viruses could modulate target cell susceptibility to lysis by NK cells. It should be noted that in the majority of those studies, the target cells were infected from 1 to 3 days, depending on the virus. It is certainly possible that as the course of certain virus infections progressed, targets might have become more sensitive to lysis by NK cells during a discrete period of time post-infection and, due to the assay conditions, this "window of vulnerability" was missed. To address this question, experiments were designed in order to avoid overt IFN-induced effects upon the effector cells, which allowed me to be confident that any alterations in target cell susceptibility to lysis by various cytotoxic effector cells were, in fact, due to the virus infection of the target itself.

A number of different viruses were tested. However, VV was used predominantly for several reasons: a) previous evidence in the laboratory suggested that VV was an NK-sensitive virus *in vivo* (Bukowski et al., 1983, 1987); b) of the other cytopathic viruses which can infect mouse fibroblasts, the kinetics of the cytopathic

effect of this virus were slower than with others (e.g., HSV-1, VSV); c) VV-specific CTL are easy to generate and examine in this system.

In order to determine if VV-infected cells were more suscep-tible to killing by NK cells, time course studies were performed. Activated NK cells from C3H/HeSnJ mice, 3 days following LCMV infection, were added in short 4-h assays to L929 cells that had been infected with VV (MOI=10) at various time intervals. Figure 5 shows the results of 4 different experiments. NK cell-mediated killing of VV-infected L929 cells increased as the infection progressed, reaching a peak by 24 - 28 h post-infection, after which time it began to decline. At later stages of infection, however, the target cells became more resistant to lysis by NK cells, consistent with results previously reported (Welsh and Hallenbeck, 1980; Welsh et al., 1989). Lysis of target cells by endogenous, nonactivated spleen cells averaged 5% or less at all time intervals examined (data not shown). These results suggest that VV-infected targets are not susceptible to lysis by non-activated NK cells, but do have a discrete "window of vulnerability" to lysis by activated NK cells. This window of vulnerability to lysis was observed whether (experiments 2 - 4) or not (experiment 1) the target cells were exposed to trypsin before the cytotoxicity assay.
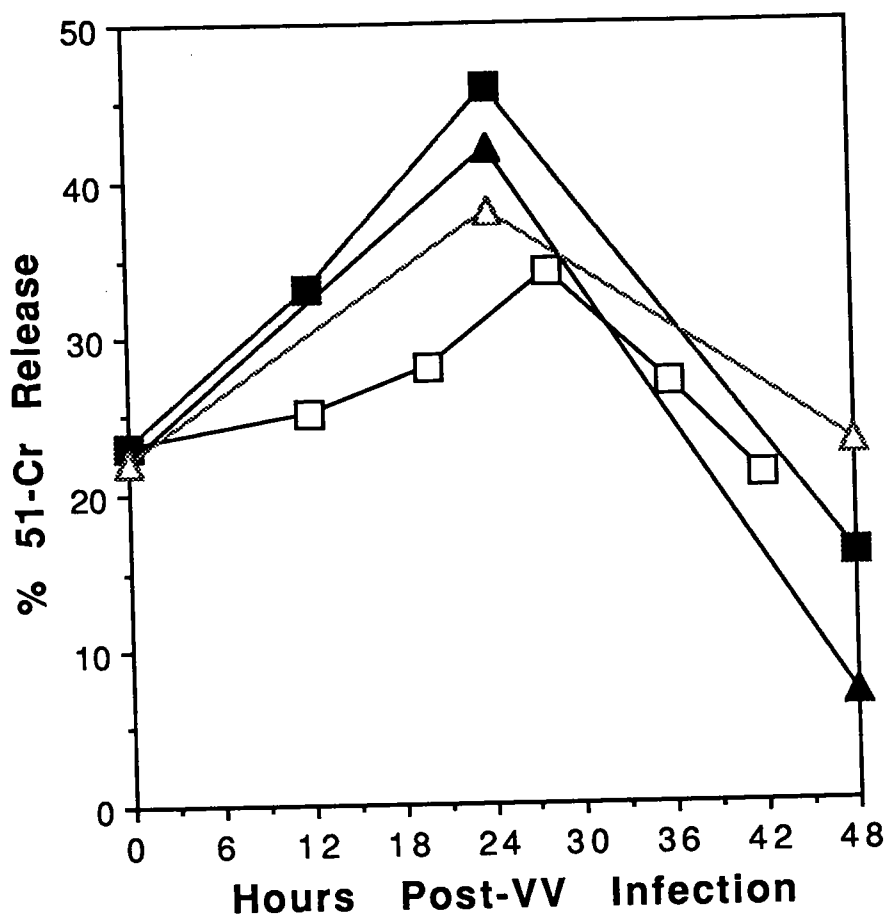
Figure 5. "Window of vulnerability" of VV-infected L929 cells to lysis by activated NK cells. L929 cells were infected with VV (MOI = 10 PFU/cell) for various intervals and served as targets of LCMV-induced, activated spleen cells from C3H/HeSnJ mice in a 4-h ^{51}Cr release assay. Four individual experiments are presented. E:T ratio = 150:1. Experiment 1 (□) was performed using ^{51}Cr prelabelled targets, whereas experiments 2 - 4 used target cells which were infected with VV before ^{51}Cr labelling. The E:T ratio in experiment 1 was based on the target cell number at the time of labelling with ^{51}Cr, which was approximately 48 h before the cytotoxicity assay; therefore, due to cell division in the microwells, the actual E:T ratio may be <150:1.

B. Effect of VV Infection on Effector/Target Cell Conjugates

In order to determine if the "window of vulnerability" to NK cell-mediated lysis was due to an increase in the number of effector/target cell conjugates, L929 cells at various time intervals after VV infection were mixed at an E:T ratio of 5:1 with LCMV-induced, activated C3H/HeSnJ spleen cells that had been depleted of most B cells, macrophages, and granulocytes by passage over nylon wool. As shown in Table 2, whereas 43% of the uninfected L929 cells bound lymphocytes, as the VV infection progressed, that number steadily declined from 19% at 24 h post-infection to 11% by 48 h. These results suggest that the observed "window of vulnerability" to NK cells was not due to increased binding of effectors to the target cells, but, given the enhanced levels of lysis, the efficiency of a bound effector cell to lyse a target must be markedly enhanced. This low level of binding at later stages of infection could have contributed to the observed resistance of targets to lysis by NK cells.

C. Optimization and Analysis of NK Cell Triggering

A critical post-binding step in the process leading to the lysis of a target cell by NK cells is the triggering of the effector cells (Welsh, 1986). In order to measure triggering, I adapted an assay which measures the uptake of [$^{45}Ca^{2+}$] into human NK cells (Jondal et al., 1986; Grönberg et al., 1988) for the study of murine NK cells. The

TABLE 2

Binding of NK Cells to VV-Infected L929 Cells[a]

Hours Post-VV Infection[b]	% Bound Targets[c]
0	43
14	36
24	19
39	12
48	11

[a]Nylon wool-passed spleen cells from C3H/HeSnJ mice were obtained 3 days post-LCMV infection and used at an E:T ratio of 5:1.

[b]L929 cells were infected with VV at an MOI of 10.

[c]Effector/target cell conjugates were enumerated after 30 min - 2 h at 4°C.

NK-sensitive YAC-1 lymphoma and L929 cells, which are less NK-sensitive, were initially compared. As shown in Table 3, exp. 1, $[^{45}Ca^{2+}]$ influx into PEC NK cells derived from MHV-infected C57BL/6 mice was considerably greater when YAC-1, rather than L929, targets were used. I also utilized activated spleen cells from LCMV-infected SCID mice as effectors in this type of assay using YAC-1 targets. SCID mice have no functional T or B cells (Bosma et al., 1983), yet have normal NK cell function (Dorshkind et al., 1985; Welsh et al., 1991). The spleens, therefore, have a high frequency of NK cells. In order to optimize conditions for SCID mouse NK cells, different E:T ratios were used. As is indicated (Table 3, exp. 2), the maximum $[^{45}Ca^{2+}]$ influx occurred at an E:T ratio of 1:1, which would enable the majority of the NK cells to find a target and be triggered.

YAC-1 and L929 target cells were treated with IFNβ or cycloheximide and tested in the triggering assays. Treatment of cells with IFN has previously been shown to upregulate the expression of MHC class I molecules (Lindahl et al., 1976; Bukowski and Welsh, 1986a) and decrease their sensitivity to lysis by NK cells by inhibiting the triggering process (Trinchieri and Santoli, 1978; Moore et al., 1980; Welsh and Hallenbeck, 1980; Welsh et al., 1981; Piontek et al., 1985; Grönberg et al., 1988). IFNβ-treated YAC-1 cells induced less of a $[^{45}Ca^{2+}]$ influx into PEC NK cells from MHV-infected mice than did untreated YAC-1 cells (Table 3, exp. 3). Cycloheximide treatment of L929 cells increases their susceptibility to NK cell-mediated cytolysis (Kunkel and Welsh, 1981), and decreases the

expression of H-2 class I molecules (Reichner et al., 1988). In contrast to that observed with the $IFN\beta$-treated YAC-1 cells, cyclo-heximide-treated L929 cells induced a greater $[^{45}Ca^{2+}]$ influx into PEC NK cells (Table 3, exp. 4). These results demonstrate reliability in the triggering assay and correlations between triggering, enhanced cytotoxicity, and reduced H-2 class I antigen expression.

D. Triggering of NK Cells during the "Window of Vulnerability"

To determine whether the increased susceptibility of VV-infected target cells to NK cell-mediated cytolysis during the "window of vulnerability" was due to an enhanced triggering of the effector cells, $[^{45}Ca^{2+}]$ uptake was measured in cocultures of LCMV-induced, activated SCID mouse splenic NK cells and L929 cells at 0, 24 and 48 h post-VV infection. As shown in the 2 experiments presented in Figure 6, the net $[^{45}Ca^{2+}]$ influx into activated SCID NK cells cocultured with uninfected L929 cells was low, peaked at 24 h post-VV infection, and by 48 h was at or below that seen with unin-fected targets. These data indicate that the "window of vulnerability" of VV-infected targets to lysis by NK cells is correlated with an enhanced triggering of the effector cells and is the first such observation in a virus infection model in the absence of increased binding.

TABLE 3

Analysis of NK Cell Triggering by $[^{45}Ca^{2+}]$ Uptake

Exp.[a]	Cell Population[b]	Total $[^{45}Ca^{2+}]$[c] (cpm)	Net $[^{45}Ca^{2+}]$ Influx (cpm)
1	PEC-NK	13,905	
	YAC-1	1,314	
	L929	1,546	
	PEC-NK + YAC-1	74,634	59,415
	PEC-NK + L929	27,507	12,056
2	SCID NK (8 x 10^5 cells)	2,457	
	YAC-1 (8 x 10^4 cells)	1,349	
	(8 x 10^5 cells)	3,951	
	NK + YAC-1 (E:T=10:1)	3,914	108
	(E:T= 1:1)	10,797	4,389
3	PEC-NK	7,203	
	YAC-1	4,868	
	YAC-1 + IFNβ[d]	4,387	
	PEC-NK + YAC-1	68,632	56,561
	PEC-NK + YAC-1/IFNβ	45,869	34,279
4	PEC-NK	12,452	
	L929	790	
	L929 + cycloheximide	3,567	
	PEC-NK + L929	18,901	5,659
	PEC-NK + L929/cyclo.	26,093	10,074

[a]Standard $[^{45}Ca^{2+}]$ influx assay with 5 x 10^6 effectors and 5 x 10^5 targets unless indicated otherwise.

[b]Effector cells were either PEC from MHV-infected C57BL/6 mice or were splenocytes from SCID (CB.17 background) mice. E:T ratios are at 10:1 except where indicated.

[c]Background (in cpm) was subtracted.

[d]YAC-1 cells were treated with 1000 U/ml of murine IFNβ for 20 h at 37°C.
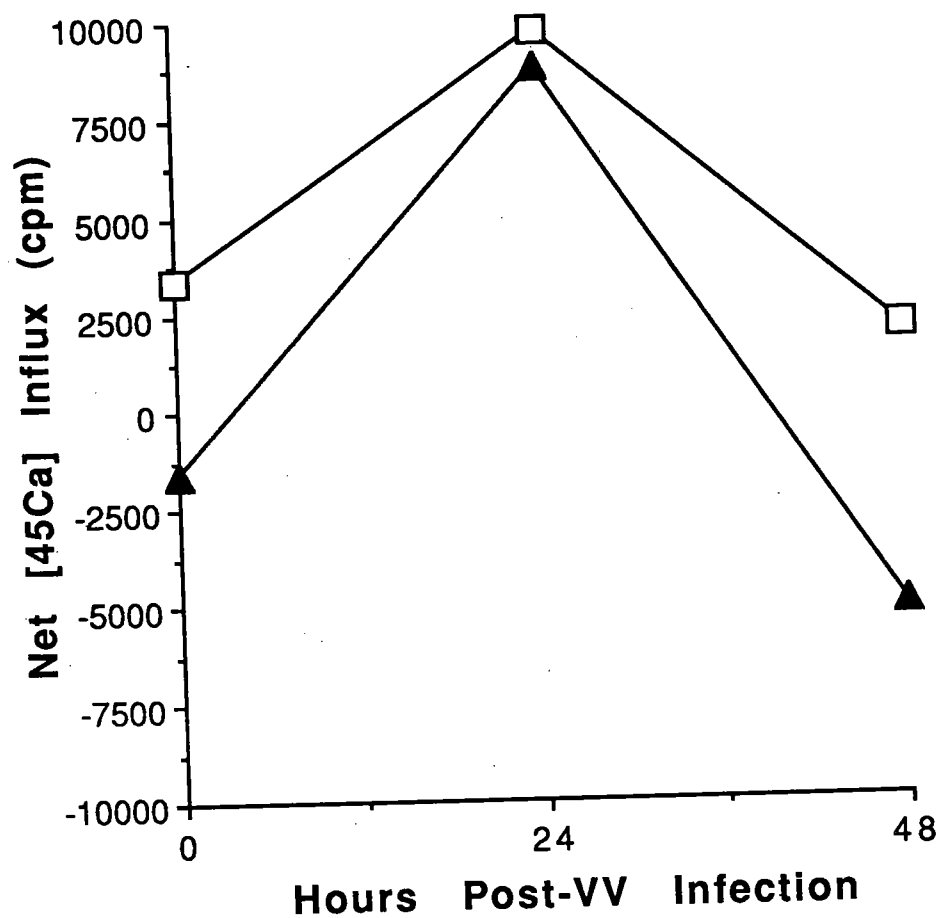
Figure 6. "Window of vulnerability" of VV-infected L929 cells correlates with enhanced triggering of NK cells. L929 cells at 0, 24, and 48 h post-VV infection were cocultured with activated SCID mouse NK cells in a micro-$[^{45}Ca^{2+}]$ influx assay. The net $[^{45}Ca^{2+}]$ influx in two different experiments is presented.

CHAPTER IV

STUDIES ON VV-INDUCED ALTERATIONS OF TARGET CELL H-2 CLASS I MOLECULES AND SUSCEPTIBILITY TO CTL-MEDIATED LYSIS

A. H-2 Class I Surface Antigen Expression on Target Cells Following VV Infection

Because inverse correlations between the expression of target cell class I MHC antigens and sensitivity to or triggering of NK cells have been noted in several systems (Ljunggren and Kärre, 1990), cell surface $H-2K^k$ and $-D^k$ class I molecules on L929 cells at various intervals post-VV infection (MOI=10) were monitored serologically by FACS. As shown in Figure 7, $H-2K^k$ and $H-2D^k$ expression of VV-infected L929 cells appeared to decrease (2-fold and 5-fold, respectively) at 24 h post-VV infection. Later in VV infection, an increase in cell background with the FITC-labelled second antibody made the interpretation difficult (data not shown). These results suggest that alterations in the expression of H-2 class I antigen expression might play a role in the enhanced sensitivity of VV-infected L929 cells to NK cell-mediated lysis.

B. "Window of Vulnerability" to NK Cell-Mediated Lysis is Concomitant with a Decrease in Susceptibility to Killing by *In Vivo*-Generated Allospecific CTL

Because I found that H-2 class I expression was decreased following VV infection of L929 cells, the lysis of VV-infected cells by

H-2 class I-restricted allospecific CTL was examined. Figure 8 compares the sensitivity of L929 cells (H-2^k) at various time points after VV infection to killing by VV-specific CTL and by *in vivo*-generated allospecific (anti-H-2^k) CTL in 3 different experiments. It is evident that as the VV infection progressed, susceptibility to lysis by VV-specific CTL increased, reached a peak at 24 h and subsequently declined. In contrast, sensitivity to killing of uninfected L929 cells by *in vivo*-generated allospecific CTL was high, but continually decreased throughout the VV infection of the target cells. These data indicate that when VV-infected cells were more susceptible to killing by NK cells (Fig. 5) and VV-specific CTL during a discrete window of time post-infection, they had become resistant to lysis by allospecific CTL.

C. Affinity of *In Vivo*-Generated Allospecific CTL

One argument that might explain the results described above would be that the allospecific CTL were of low affinity compared to the high affinity VV-specific CTL, obtained 6 or 7 days post-infection. In order to determine if the *in vivo*-generated allospecific CTL were of high affinity, several serial dilutions of anti-Lyt 2 (CD8) antibody were included in a 5-h cytotoxicity assay. As shown in Figure 9, the highest antibody concentrations used inhibited only about 50% of the lysis mediated by allospecific CTL, indicating that at least half of the
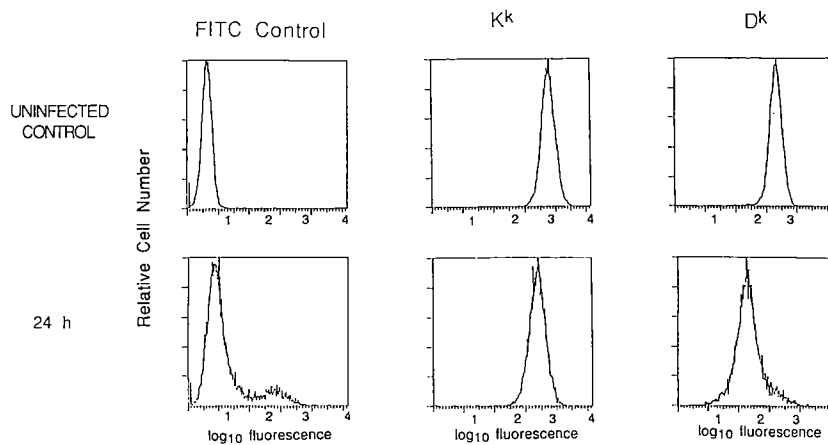
Figure 7. Cell surface expression of H-2K^k and H-2D^k on L929 cells following VV infection. L929 cells (uninfected or 24 h post-VV infection) were labelled with K^k- or D^k-specific monoclonal alloantibodies followed by a FITC-conjugated goat anti-mouse Ig antiserum, and expression of K^k and D^k were analyzed by FACS.
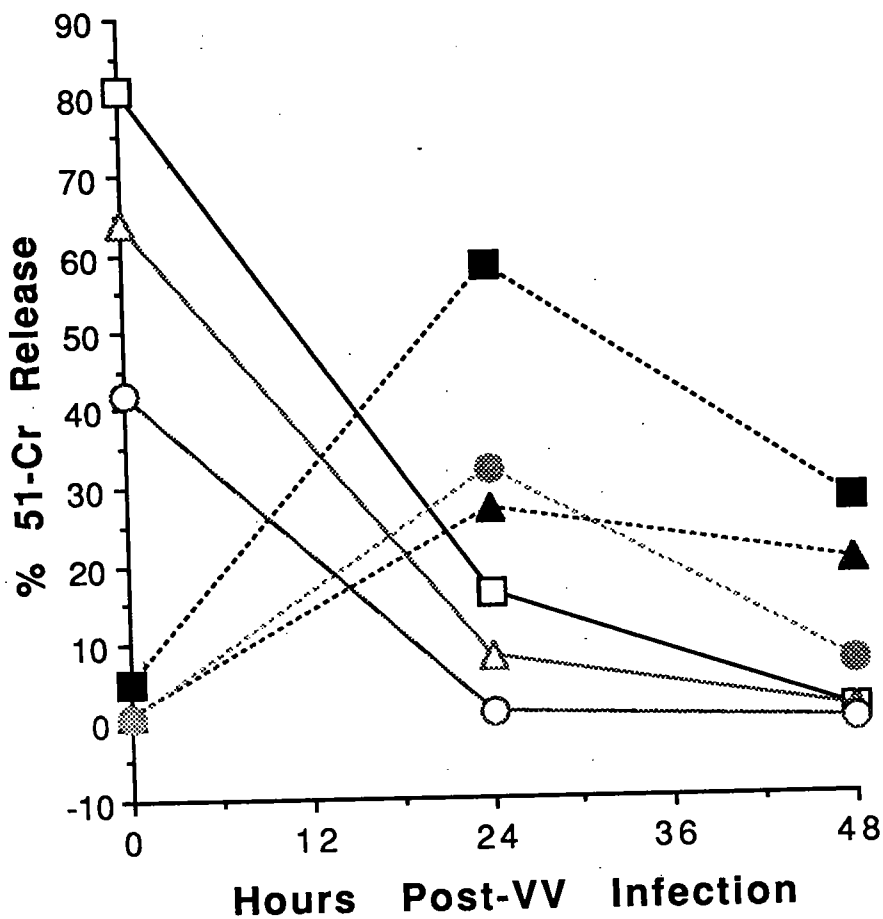
Figure 8. Sensitivity of VV-infected L929 cells to killing by VV-specific CTL and anti-H-2^k allospecific CTL. L929 cells were used as targets in 4-h ^{51}Cr release assays at various intervals after VV infection for VV-specific (---) or *in vivo*-generated allospecific (—) CTL. Three individual experiments are presented (corresponding closed and open symbols). E:T ratios = 100:1 for VV-specific, 50:1 for allospecific CTL.
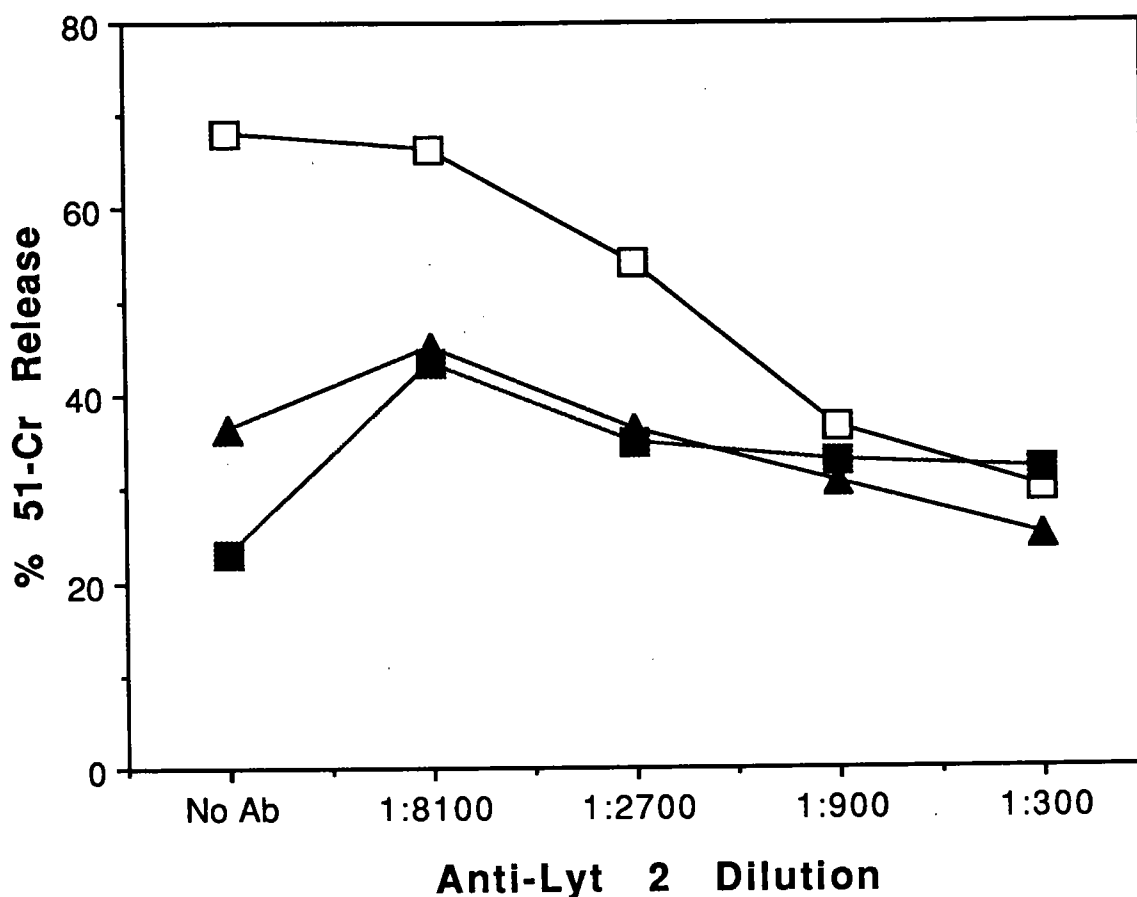
Figure 9. Anti-Lyt 2 antibody-mediated inhibition of allospecific (anti-H-2^k) and VV-specific CTL. Various dilutions of anti-Lyt 2 mAb were added to wells containing L929 cells (uninfected for allospecific CTL or 24 h post-VV infection for VV-specific CTL). Percent ^{51}Cr release was determined in a 5-h ^{51}Cr release assay. E:T ratios: allospecific CTL, 50:1; VV-specific CTL, 100:1. Symbols: allospecific CTL (□); day +6 VV-specific CTL (▲); day +7 VV-specific CTL (■).

allospecific killing was mediated by CTL of sufficiently high affinity to not require CD8-mediated adhesion for cytotoxic function.

D. Killing of L929 Cells by Anti-K^k and Anti-D^k Allospecific CTL

The previous experiments showed that not only were the VV-infected target cells resistant to killing by anti-H-2^k allospecific CTL, but also illustrated differences in the down-regulation of H-$2K^k$ and H-$2D^k$ expression following VV infection of L929 cells. As CTL responses against L929 cells (H-2^k) are restricted by H-$2K^k$ or H-$2D^k$ antigens (Blanden et al., 1975), it was possible that the loss in susceptibility of VV-infected target cells to lysis by allospecific CTL was more pronounced in those specific for K^k or D^k. To investigate this possibility, anti-K^k and -D^k allospecific CTL were generated in A/J (K^k D^d) and C3H.OH (K^d D^k) mice, respectively. As Figure 10a illustrates, anti-H-$2K^k$ allospecific CTL, derived from BALB/c (H-2^d) spleen cells and generated in irradiated A/J recipient mice, efficiently lysed uninfected L929 cells. At 24 h post-VV infection, target cell susceptibility to these effector cells was markedly decreased and completely abolished by 48 h. They were, however, sensitive to VV-specific CTL. As was observed for K^k-specific CTL, anti-D^k allospecific CTL killed uninfected L929 cells and VV infection of the target cells abrogated their susceptibility to killing by these effectors (Figure 10b). Other targets with different allotypes were used to ensure that the killing when present was due to an

allospecific CTL phenomenon and not to other cytotoxic cells (not shown). These results indicate that the sensitivity to killing by both anti-K^k and -D^k allospecific CTL is decreased in L929 cells following VV infection. To be sure that the VV-specific CTL were recognizing the L929 (H-2^k) targets appropriately, these effector cells were also generated in A/J (K^k D^d) and C3H.OH (K^d D^k) mice and compared to those obtained from C3H/HeSnJ (K^k D^k) mice. As shown in Figure 11 and reported elsewhere (Zinkernagel and Doherty, 1979; Müllbacher et al., 1989), the bulk of the anti-VV response was K^k-restricted.
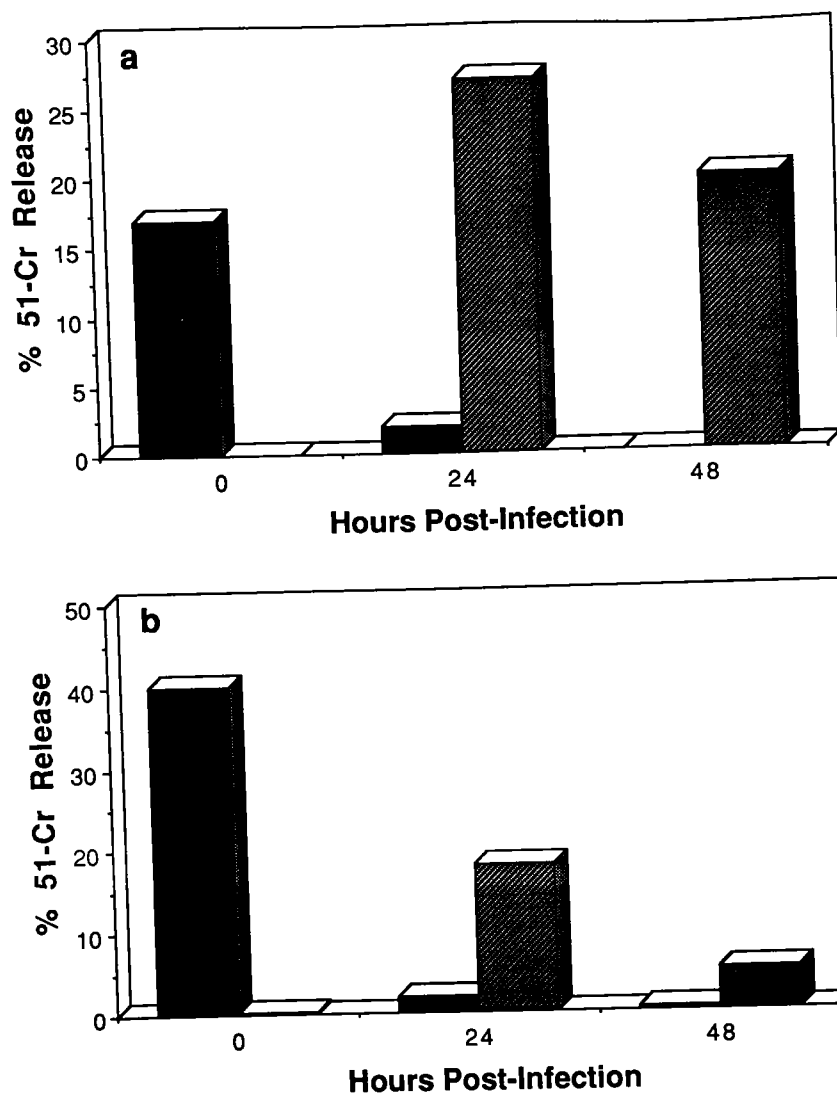
Figure 10. Sensitivity of VV-infected L929 cells to lysis by anti-H-2K^k (a) or -H-2D^k (b) allospecific CTL. In **a**, allospecific CTL generated in A/J mice (H-2K^{k}D^d, solid bar) were compared to VV-specific CTL obtained from C3H/HeSnJ mice (H-2K^{k}D^k, shaded bar). E:T ratios were 50:1 for allospecific CTL, 100:1 for VV-specific CTL. In **b**, allospecific CTL were generated in C3H.OH mice (H-2K^{d}D^k, solid bar). The other effectors are as in **a**. L929 cells were used as targets in a 5-h ^{51}Cr release assay at various intervals post-VV infection (MOI = 10). E:T ratio was 50:1 for all effectors in **b**.
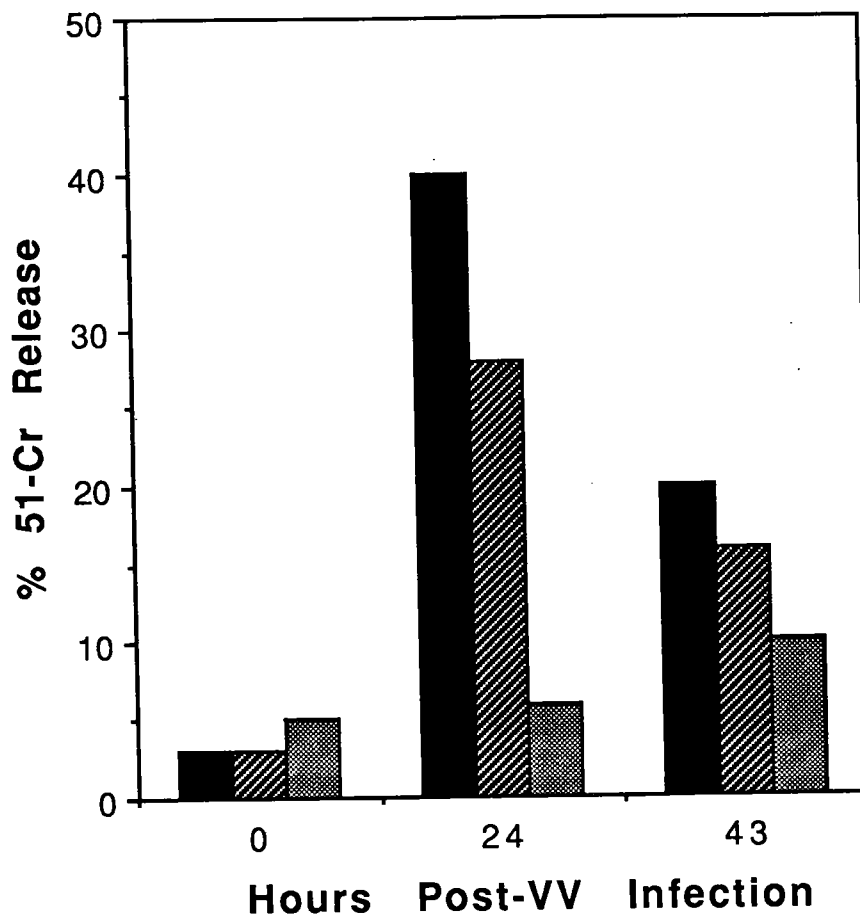
Figure 11.   Demonstration that the bulk of VV-CTL activity against VV-infected L929 cells is K^k-restricted.   C3H/HeSnJ (H-2K^k, D^k; black bars), A/J (H-2K^k, D^d; striped bars), and C3H.OH (H-2K^d, D^k; grey bars) mice were infected i.p. with 2 x 10^6 PFU of VV.   Six days later, spleens were harvested and used as effectors against uninfected L929 cells or those infected with VV for 24 or 43 hours in a 5-h ^{51}Cr release assay.   MC57G cells (H-2^b) served as a negative control.   E:T ratio = 100:1.

E. Sensitivity of L929 H-2 Class I Variant
Cell Lines to Lysis by Allospecific CTL

I showed above that there was both a reduction in H-2 class I antigen expression on L929 cells following VV infection, in addition to enhanced resistance to killing by allospecific CTL. In order to determine if this reduction in the levels of H-2 class I molecules after VV infection was sufficient to abrogate target cell susceptibility to allospecific CTL-mediated lysis, L929 variant cells which expressed lower levels of H-2 class I antigens were used. As shown in Figure 12, 4a-1:3 cells expressed lower levels of H-2 class I antigens (2-fold) as compared to normal L929 cells. However, in contrast to that found in VV-infected targets, the L929 variant cells were still killed by allospecific CTL at high levels. Whereas 74% lysis [19.9 lytic units (LU)/10^6 cells] of normal L929 cells was observed using allospecific CTL at a 50:1 E:T ratio, 48% lysis (7.9 LU/10^6 cells) was obtained when the L929 variant cells were used as target cells (one LU represents the number of effector cells required to lyse 25% of the target cells). Similar results were found with another variant line, 5a-15:9 (62% lysis, 10.5 LU/10^6 cells). Because cycloheximide inhibits protein synthesis and the reexpression of H-2 class I molecules on the cell surface (Reichner et al., 1988), L929 cells were treated with 25 μg/ml of cycloheximide for 18 - 20 h (Kunkel and Welsh, 1981) and used as targets. As shown in Figure 13, cycloheximide treatment of L929 cells resulted in a 13-fold decrease

in H-2 class I antigen expression, but these targets also remained relatively sensitive to lysis by allospecific CTL [32% lysis (3.2 LU/10^6 cells) as compared to 57% lysis (9.1 LU/10^6 cells) with untreated L929, 50:1 E:T ratio]. Although the reduction in H-2 class I molecules on L929 cells after cycloheximide treatment was substantially greater than that found after VV infection, these cells still served as adequate targets for allospecific CTL. Therefore, the decrease in H-2 class I antigen expression does not by itself explain the VV-induced abrogation of susceptibility to allospecific CTL-mediated lysis.

F. Susceptibility of L929 Cells Persistently-Infected with LCMV [L(LCMV) Cells] to Killing by VV-Specific, LCMV-Specific, or Allospecific CTL after VV Infection

The above data suggested that the modulation in the sensitivity of VV-infected target cells to lysis by NK cells or allospecific CTL may have been due to alterations in endogenous peptide presentation on H-2 class I molecules. To test this hypothesis, L(LCMV) cells were infected with VV at an MOI of 10 for various lengths of time and then used as targets in a ^{51}Cr release assay. VV- or LCMV-specific CTL were obtained from spleen cells derived from C3H/HeSnJ mice, 7 - 8 days after an i.p. infection with VV (1 x 10^7 PFU) or LCMV (5 x 10^4 PFU), respectively. *In vivo*-generated allospecific (anti-H-2^k) CTL were obtained from irradiated C3H/HeSnJ mice 5 days after receiving either BALB/c (H-2^d) or C57BL/6 (H-2^b) donor spleen cells.

As shown in Figure 14, VV-specific CTL were unable to lyse L(LCMV) cells before infection with VV. However, after VV infection, the sensitivity of L(LCMV) cells to killing by VV-specific CTL increased and peaked at 12 -24 h. Whereas L(LCMV) cells were killed by LCMV-specific CTL or by allospecific CTL before VV infection, infection of these targets with VV resulted in a rapid loss in susceptibility to lysis by either of these effector cells. These results, in addition to those described above, are consistent with the hypothesis that alterations in endogenous peptide presentation by VV-infected targets may have affected target sensitivity to lysis by allospecific (or LCMV-specific) CTL, and possibly even by NK cells. As will be shown in the following chapter, however, my evidence does not support a role for peptide modification in enhancing the susceptibility of targets to lysis by NK cells.

CLASS I ANTIGEN EXPRESSION

Normal L929
LU/10^6 cells = 19.9
% Lysis = 74

4a-1:3
LU/10^6 cells = 7.9
% Lysis = 48

5a-15:9
LU/10^6 cells = 10.5
% Lysis = 62

Relative Cell Number

log$_{10}$ fluorescence
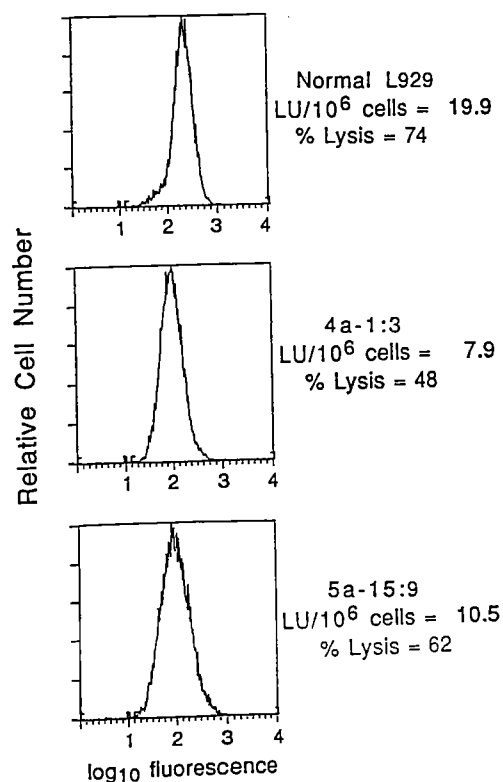
Figure 12. L929 variants expressing reduced levels of H-2 class I antigens comparable to VV-infected L929 cells are susceptible to lysis by allospecific CTL. The L929 variants, 4a-1:3 and 5a-15:9, were stained with a rat anti-mouse H-2 class I antibody, followed by a FITC-labelled mouse anti-rat antibody. Analysis was by FACS. The cells were also used as target for *in vivo*-generated allospecific CTL derived from C57BL/6 donors (C3H/HeSnJ recipients). E:T ratio = 50:1, 6-h assay. Cytotoxicity results are indicated as % lysis and lytic units (LU)/10^6 cells.

FITC CONTROL

NORMAL L929

Relative Cell Number

CYCLOHEXIMIDE-
TREATED

$\log_{10}$ fluorescence
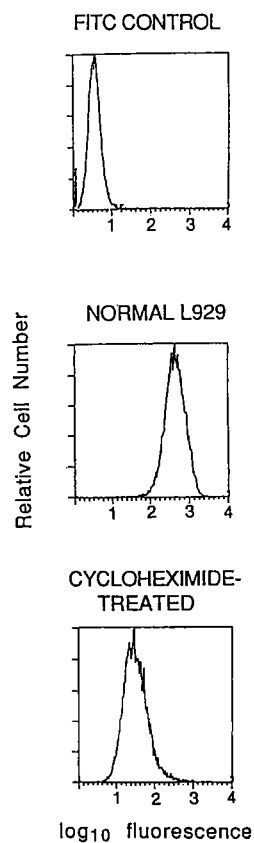
Figure 13. Cell surface expression of H-2 class I molecules on cycloheximide-treated (25 μg/ml, 19 h) L929 cells. The cells were labelled with a monoclonal rat anti-mouse H-2 class I antibody, followed by a FITC-conjugated mouse anti-rat antibody. Analysis was performed by FACS. In parallel, samples of untreated or cycloheximide-treated L929 cells were labelled with ^{51}Cr and used as target in a 6-h ^{51}Cr release assay with allospecific CTL (generated in C3H/HeSnJ mice with C57BL/6 donors) as effectors at a 50:1 E:T ratio. The results of the cytotoxicity assays are indicated in the text.
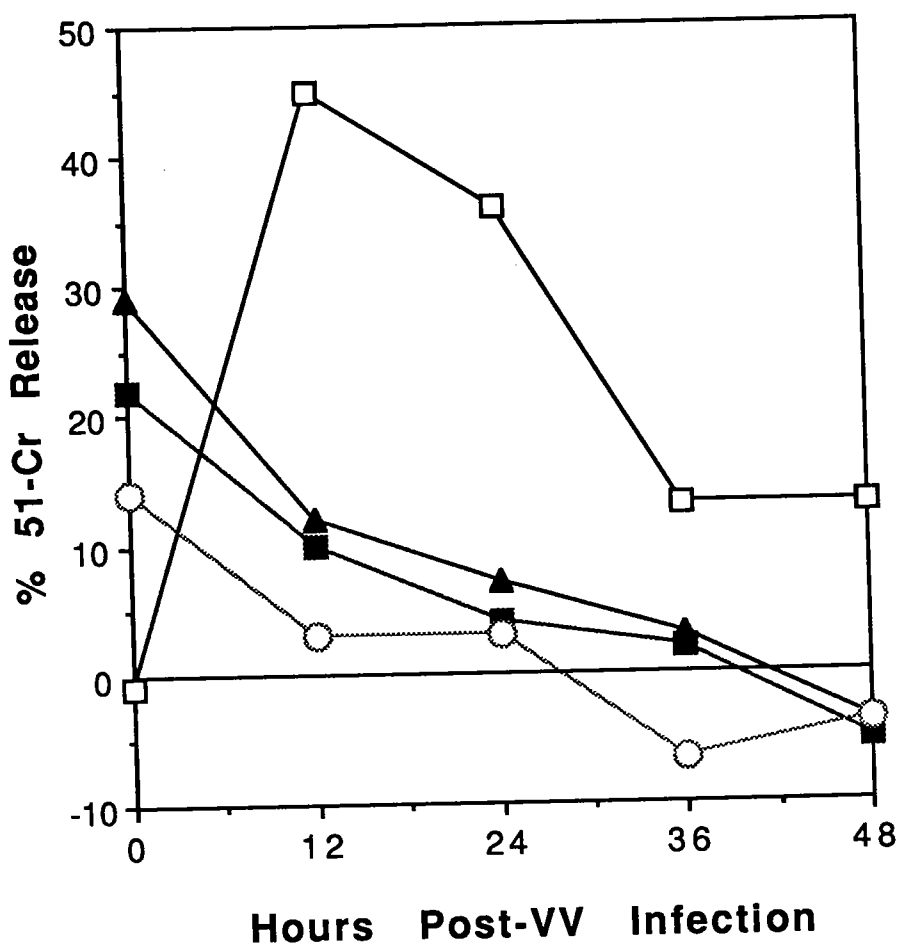
Figure 14. Susceptibility of VV-infected L(LCMV) cells to killing by CTL. L929 cells, persistently-infected with LCMV were infected with the WR strain of VV at an MOI of 10. At various times after infection, the cells were used as targets in a 4-h ^{51}Cr release assay. The following effector cells were employed at an E:T ratio of 100:1: VV-specific CTL (□), LCMV-specific CTL (▲), allospecific CTL generated in irradiated C3H/HeSnJ mice (H-2^k) with BALB/c (H-2^d) donors (■); allospecific CTL using C57BL/6 (H-2^b) donors (0).

CHAPTER V

STUDIES ON THE ROLE OF VIRAL PEPTIDE CHARGING OF H-2 CLASS I MOLECULES IN THE SUSCEPTIBILITY OF TARGET CELLS TO LYSIS BY NK CELLS

A. Overnight Treatment of Target Cells with Synthetic Viral Peptides does not Alter Sensitivity to NK Cell-Mediated Lysis

Because the results in the previous chapter were consistent with viral peptide charging of H-2 class I molecules modulating target cell susceptibility to NK cell-mediated lysis, and because of work showing that modifications in the MHC peptide-binding groove alters target cell susceptibility to lysis (Storkus et al., 1991), I decided to test this hypothesis directly. Initially, I used an *in vitro* approach in which I added synthetic peptides, corresponding to the natural or minimal immunodominant epitopes as defined for virus-specific CTL (Oldstone, 1991). Table 1 shows the peptides that were used. In the experiments shown in Table 4, target cells were pretreated overnight with 200 - 250 μM of the indicated peptides, washed and used in 4 - 6 h ^{51}Cr release assays. Influenza has been shown to be an NK-sensitive virus in mice and hamsters (Stein-Streilein and Guffee, 1986; Guffee et al., 1989). In the representative experiments shown in Table 4, no significant difference in the sensitivity of targets pulsed overnight with 3 different influenza peptides, which are presented on different H-2 class I molecules, to NK cell-mediated lysis, compared to targets treated with no peptide. Furthermore, neither the L^d-restricted IEpp89 immunodominant

TABLE 4

Effect of Overnight Preincubation with Synthetic Immunodominant
Viral Peptides on Target Cell Susceptibility to NK Cell-Mediated Lysis[a]

| Peptide (RE)[c] | Target Cell (Haplotype) | % Lysis by NK Cells[b] | | | | | |
| | | No Peptide | | | Peptide Pretreatment | | |
		100:1	50:1	25:1	100:1	50:1	25:1
HIV-1-IIIB gp160 (D^d)	18.81 (H-2^d)	41	32	24	41	31	21
HSV-1 gB (K^b)	P52 (H-2^b)	11	7	5	9	7	4
Influenza HA (K^d)^d	18.81 (H-2^d)	64	54	40	57	47	40
Influenza HA (K^d)^e	P815 (H-2^d)	ND^f	-0.8	ND	ND	-1.8	ND
Influenza NP (D^b)	P52 (H-2^b)	58	46	38	61	50	38
Influenza NP (K^k)	R1.1 (H-2^k)^g	52	41	30	53	44	36
LCMV NP (D^b)	P52 (H-2^b)	17	11	7	17	15	11
LCMV NP (D^b)^h	P52 (H-2^b)	12	11	7	11	11	8
LCMV NP (L^d)	L-L^d (H-2^k + H-2L^d)	21	14	10	20	13	9
MCMV IEpp89 (L^d)	L-L^d (H-2^k + H-2L^d)	21	14	10	18	12	7
Rabies NS (K^k)	R1.1 (H-2^k)^g	32	25	16	33	25	21
VSV N (K^b)	P52 (H-2^b)	21	13	10	18	11	6

[a]Indicated target cells were preincubated with or without 200 - 250 µM of peptide for 16 - 18 h with or without ^{51}Cr at 37°C, except where noted. Otherwise, targets were labelled with ^{51}Cr for 90 min following overnight incubation with peptide. Targets were then washed and used in 4 - 6 h ^{51}Cr release assays.

[b]Percent lysis at the indicated E:T ratios by activated splenic NK cells from mice of the appropriate haplotype.

cFor peptide sequences used, see Table 1. RE = H-2 class I presenting molecule.

dInfluenza HA$_{147-161}$ peptide.

eInfluenza HA$_{515-526}$ peptide. Peptide pretreatment for 90 min during ^{51}Cr labelling.

fNot done.

gLabelled with ^{51}Cr following overnight incubation with peptide.

hC57BL/6 LAK cell effectors.

peptide from the prototypic NK-sensitive virus, MCMV, nor the minimal immunodominant peptide from VSV N (K^b-restricted), were able to modulate susceptibility of the indicated targets to lysis by NK cells. In order to demonstrate that these peptides were indeed charging the appropriate H-2 class I molecules, primary virus-specific CTL were generated by infecting mice of the appropriate haplotype with the appropriate virus and harvesting spleen cells 6 - 8 days post-infection for use as effectors in the assays. Virus-specific CTL were not generated against all of the viral peptides because some primary CTL are difficult, if not impossible, to induce *in vivo* for testing *in vitro*. However, I was able to use either primary virus-specific CTL or peptide-specific CTL clones for most of the peptides. For example, as shown in Table 5A, 18.81 ($H-2^d$) pre-B cells prepulsed with the K^d-restricted influenza $HA_{515-526}$ peptide and then washed, were killed quite well by the CTL clone, B7-B7, which is specific for this peptide. Additionally, primary CTL induced by VSV were able to efficiently kill P52 ($H-2^b$) cells, but only after the targets were pretreated with the appropriate peptide (Table 5A).

Peptides corresponding to defined immunodominant epitopes from HSV-1 gB (K^b-restricted) and LCMV NP (D^b- and L^d-restricted) were also tested. LCMV has been defined as an NK-resistant virus in mice (Bukowski et al., 1983). Although some evidence suggests that HSV-1 is NK-sensitive in humans (Santoli et al., 1978a, b; Fitzgerald et al., 1982; Bishop et al., 1983, 1984) and mice (Lopez et al., 1980; Habu et al., 1984), our laboratory has reported that NK cells are not

TABLE 5

Effect of Synthetic Immunodominant Viral Peptides on Sensitivity of Targets to Lysis by Primary Virus-Specific CTL or Peptide-Specific CTL Clones

Peptide[a] (RE)	Target Cells (Haplotype)	Effector Cells	E:T Ratio	% Lysis of Targets	
				No Peptide	With Peptide
A. Preincubation					
Influenza HA515-526 (K^d)	P815^b	B7-B7 clone	10:1	-1.2	11
LCMV NP^c (D^b)	P52 (H-2^b)	LCMV-CTL^d (d+8 post-inf.)	100:1 50:1 25:1	2 0.6 0.0	55 43 24
LCMV NP (L^d)	L-L^d (H-2^k + L^d)	LCMV-CTL^e (d+7 post-inf.)	100:1 50:1 25:1	3 2 2	13 9 5
LCMV NP (L^d)	P815 (H-2^d)	LCMV-CTL^e (d+8 post-inf.)	100:1 50:1 25:1	-0.7 -0.2 0.5	12 6 3
HIV-1 IIIB gp160 (D^d)	18.81 cells (H-2^d)	gp160 CTL^f (d+7 post-inf.)	100:1 50:1 25:1	3 3 2	22 14 10
B. In Assay					
Influenza HA515-526 (K^d)	Con A blasts (H-2^d)	B7-B7 clone	15:1	2.5	49

B. In Assay (con't)

Peptide[a] (RE)	Target Cells (Haplotype)	Effector Cells	E:T Ratio	% Lysis of Targets	
				No Peptide	With Peptide
Influenza HA515-526 (K^d)	18.81 cells ($H-2^d$)	B7-B7 clone	10:1	-2.4	26
LCMV NP (L^d)	P815 cells ($H-2^d$)	LCMV-CTL[e] (d+8 post-inf.)	100:1	-1.3	22
			50:1	0.0	21
			25:1	1	12
VSV N (K^b)	P52 cells ($H-2^b$)	VSV-CTL[d] (d+6 post-inf.)	100:1	0.5	18
			50:1	0.2	11
			25:1	0.0	7
LCMV NP (D^b)	L-D^b ($H-2^k + D^b$)	LCMV-CTL[d] (d+7 post-inf.)	100:1	6	39
			50:1	4	30
			25:1	2	21

[a]Peptides were added to ^{51}Cr-labelled targets and incubated overnight at 200 - 250 µg/ml (except where noted) and washed (A) or added to wells of a 96 well plate without effectors for 30 min at 37°C (B). The indicated CTL effector cells were then added at various E:T ratios for a 6-h ^{51}Cr release assay (final peptide concentration=10 µg/ml). RE = H-2 class I presenting molecule.

[b]Preincubated with targets during ^{51}Cr labelling period (90 min).

[c]Target cells were preincubated with 500 µg/ml peptide overnight.

[d]CTL generated in LCMV-infected C57BL/6 mice.

[e]CTL generated in LCMV-infected BALB/c mice.

[f]gp160 CTL generated in BALB/c mice infected i.p. with VV-abtΔ74-79.

required to regulate this infection in C57BL/6 mice (Bukowski and Welsh, 1986b). The HSV-1 and LCMV peptides did not significantly alter the sensitivity of the target cells to NK cell-mediated lysis (Table 4). As shown in Table 5A, LCMV-specific CTL from either H-2^b (C57BL/6) or H-2^d (BALB/c) mice were able to kill targets prepulsed overnight with the appropriate D^b- or L^d-restricted LCMV peptides, respectively.

One other peptide derived from sequences from a virus with unknown NK-sensitivity (rabies NS, K^k-restricted) and one from the HIV-1 (IIIB) gp160 (D^d-restricted), were also evaluated. These peptides were also unable to affect the susceptibility of the targets to NK cell-mediated lysis (Table 4). To demonstrate that the HIV-1 (IIIB) D^d-restricted peptide was indeed charging the appropriate H-2 class I molecules, gp160-specific CTL were induced by infecting BALB/c (H-2^d) mice with the recombinant VV, VV-abtΔ74-79, which expresses a truncated form of the HIV-1 (IIIB) envelope gene. As Table 5A shows, only 18.81 cells treated with the HIV-1 peptide were killed by these CTL. Therefore, these results suggest that viral peptide charging of H-2 class I molecules by overnight pretreatment does not modulate target cell sensitivity to lysis by NK cells.

B. **Synthetic Viral Peptides Present throughout the Assay do not Modulate Target Cell Susceptibility to Lysis by NK Cells**

While my studies were underway, two reports from the same laboratory (Chadwick and Miller, 1992; Chadwick et al., 1992), suggested that when peptides (10 µg/ml) were present during the entire 6-h ^{51}Cr release assay following a 30 min preincubation before adding effector cells, Con A blast target cells were more sensitive to lysis by NK cells than those in the absence of peptide. Using Con A blasts or other cell lines as targets, I was unable to find that these targets were more sensitive to lysis by NK cells when peptides were present throughout the assay at a final concentration of 10 µg/ml (Table 6). For example, as shown in Table 6, neither BALB/c splenic Con A blasts, 18.81 (H-2^d) or P815 (H-2^d) target cells differed in their sensitivity to lysis by NK cells in the presence of the influenza HA$_{515-526}$ (K^d-restricted) peptide. As expected, these targets were sensitive to lysis by the CTL clone, B7-B7, when that peptide was present (Table 5A). Similar results were observed with the VSV peptide and other immunodominant peptides (Tables 5 and 6). In the reports referred to above, the authors used IL-2-activated spleen cells, also known as LAK cells. When LAK cells were obtained from C57BL/6 mice and used as effectors against the LCMV NP (D^b-restricted) peptide, no difference was detected in the sensitivity of the P52 targets to lysis by these effector cells (Table 4). LAK cells generated from SCID (H-2^d) mice are entirely NK cells, due to the

absence of T or B cells in these mice (Bosma et al., 1983). Therefore, SCID LAK cells were used as effectors in assays where the influenza $HA_{515-526}$ peptide (K^d-restricted) was present throughout the assay. Not surprisingly, no alterations in the susceptibility of either 18.81 or P815 (both $H-2^d$) targets to lysis by SCID LAK cells was observed in the presence of the peptide (Table 6). Over two dozen experiments with various peptide/target cell combinations with either NK or LAK cells as effectors were performed, with all giving similar negative results. The targets included: "normal" cells (i.e., Con A blasts), thymomas, mastocytomas, pre-B cells, fibroblasts, and thymomas or fibroblasts transfected with various H-2 class I molecules. Therefore, as a result of these numerous experiments, I find that even when the peptides are present throughout the assay, target cells are not more sensitive to lysis by NK cells. Furthermore, other laboratories have been attempting to modulate target cell sensitivity to NK cell-mediated lysis using synthetic viral peptides and have been unsuccessful as well (R. Kiessling, H. -G. Ljunggren, and M. Trucco, personal communications).

I should point out that in my very earliest preliminary experiments, I used the D^b-restricted LCMV NP peptide and, based on information from Dr. Michael Oldstone's laboratory, the peptide was included in the assays at 200 - 250 $\mu g/ml$. At this extremely high concentration, L-D^b or D1R cells with the NP peptide appeared to be actually more resistant to lysis by NK cells, when compared to the same targets without peptide, although the peptide-treated

targets were sensitive to lysis by LCMV-specific CTL (Figure 15). Because the studies reported above (Chadwick and Miller, 1992; Chadwick et al., 1992) used considerably less peptide in their assays, and to avoid any potential nonspecific effects, my subsequent experiments used lower concentrations.

TABLE 6

Effect of Synthetic Immunodominant Viral Peptides Present throughout Assay on Target Cell Susceptibility to NK Cell-Mediated Lysis[a]

Peptide (RE)[c]	Target Cell (Haplotype)	% Lysis by NK Cells[b]					
		No Peptide			Peptide In Assay		
		100:1	50:1	25:1	100:1	50:1	25:1
Influenza HA (Kd)[d]	Con A blasts (H-2d)[e]	-2.9	N.D.[f]	2	-1.3	N.D.	-3.6
Influenza HA (Kd)[d]	18.81 (H-2d)	14	N.D.	8	17	N.D.	11
Influenza HA (Kd)[d]	P815 (H-2d)	11	10	7	12	10	7
Influenza HA (Kd)[d]	P52 (H-2b)	13	9	6	9	5	2
VSV N (Kb)	18.81 (H-2d)	N.D.	28	N.D.	N.D.	31	N.D.
Influenza HA (Kd)[d,g]	L-Db (H-2k + Db)	18	13	7	20	13	7
LCMV GP (Db)	L-Db (H-2k + Db)	18	13	7	17	12	7
LCMV NP (Db)	P815 (H-2d)	10	9	8	10	11	9
LCMV NP (Ld)	R1.1 (H-2k)	N.D.	38	25	N.D.	36	23
Rabies NS (Kk)							

[a]Peptides were added to ^{51}Cr-labelled targets and incubated wihout effectors for 30 min at 37°C. Splenic NK cells were then added at the indicated E:T ratios for 4 - 6 h ^{51}Cr release assays (final peptide concentration = 10 μg/ml).

[b]Percent lysis at the indicated E:T ratios by activated splenic NK cells from mice of the appropriate haplotype.

[c]For peptide sequences used, see Table 1. RE = H-2 class I presenting molecule.

[d]Influenza HA$_{515-526}$ peptide.

82

^eDerived from spleens of BALB/c mice.

^fNot done.

^gCB.17 SCID LAK cell effectors.
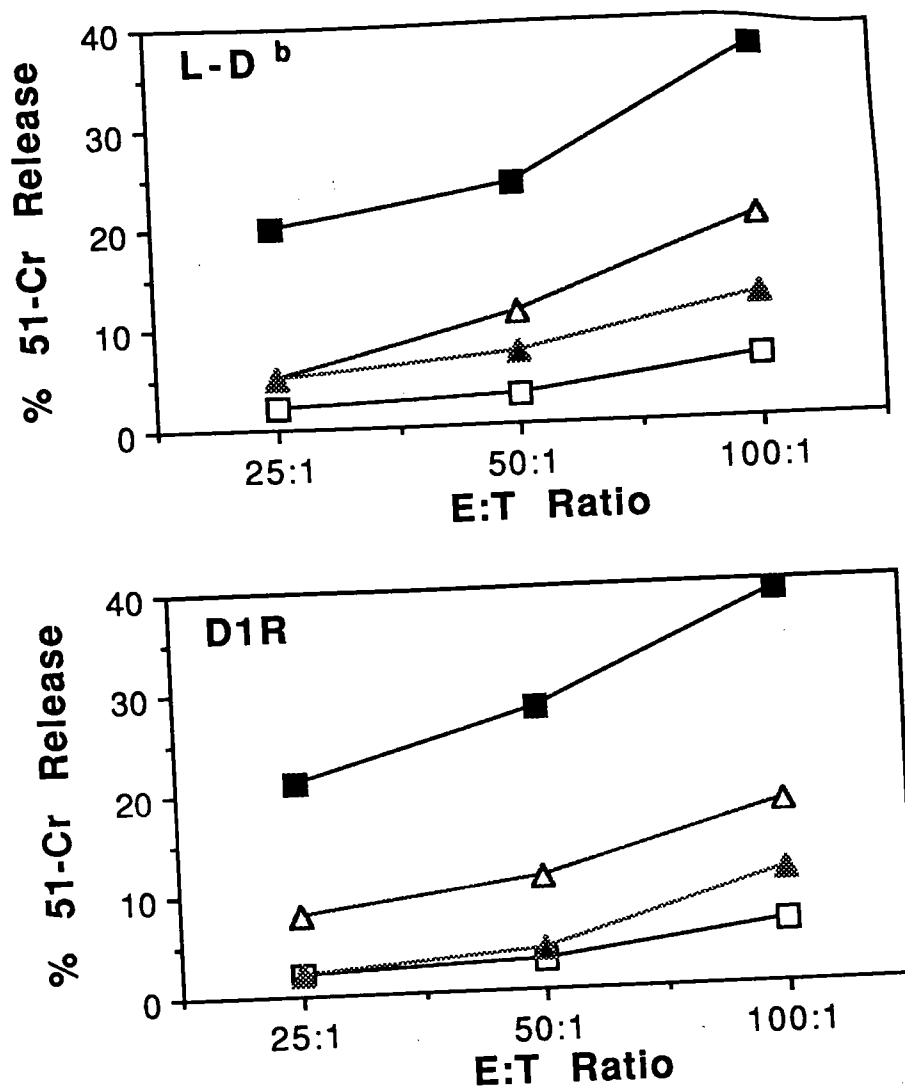
Figure 15. Effect of LCMV NP peptide on the sensitivity of L-D^b and D1R cells to NK cell-mediated lysis. ^{51}Cr-labelled L-D^b (top) and D1R (bottom) cells were plated in 96 well plates and either LCMV NP peptide (D^b-restricted) for a final concentration of 200 μg/ml (filled symbols), or medium (open symbols) were added to the appropriate wells. After 45 min at 37°C, spleen cells from C57BL/6 mice, 3 days (NK, $\triangle$) or 9 days (LCMV-CTL, $\square$) after LCMV infection were added at various E:T ratios for a 6-h ^{51}Cr release assay.

C. Saturation of Both H-2 Class I Allotypes on P52 (H-2^b)
Cells with Peptides does not Increase Target Cell
Sensitivity to Lysis by NK Cells

It is possible that in the previous experiments we failed to demonstrate a peptide-induced alteration of target cell susceptibility to NK cell-mediated lysis because we only used a single immunodominant peptide which was presented on a single H-2 class I allotype. Because saturation of both H-2 class I allotypes may have given a different result, P52 target cells were pretreated overnight with both the K^b-restricted VSV peptide and the D^b-restricted LCMV NP peptide. The cells were then labelled with ^{51}Cr, washed free of peptide, and used as target cells in a 6-h killing assay against splenic NK cells from C57BL/6 mice. In parallel, the peptides were added to targets in the assay. As shown in Figure 16, when target cells were treated overnight with both peptides and washed (Fig. 16a), or when both peptides were present throughout the assay (Fig. 16b), no change in the sensitivity of P52 target cells to lysis by NK cells was observed. Primary VSV- and LCMV-specific CTL were also generated in C57BL/6 (H-2^b) mice. As is also evident in Figure 16, these effectors were only able to lyse the targets when the the targets were prepulsed overnight with the appropriate peptide (Figure 16a) or if the peptides were present in the assay (Figure 16b). As shown in Figure 17, similar results were obtained when the K^b-restricted HSV-1 gB peptide was used with the D^b-restricted LCMV NP peptide.

LCMV-specific CTL could only kill those targets pretreated overnight with the LCMV peptide. These data further support the above results suggesting that viral peptide charging of H-2 class I molecules <u>does</u> <u>not</u> affect target cell susceptibility to NK cell-mediated lysis.
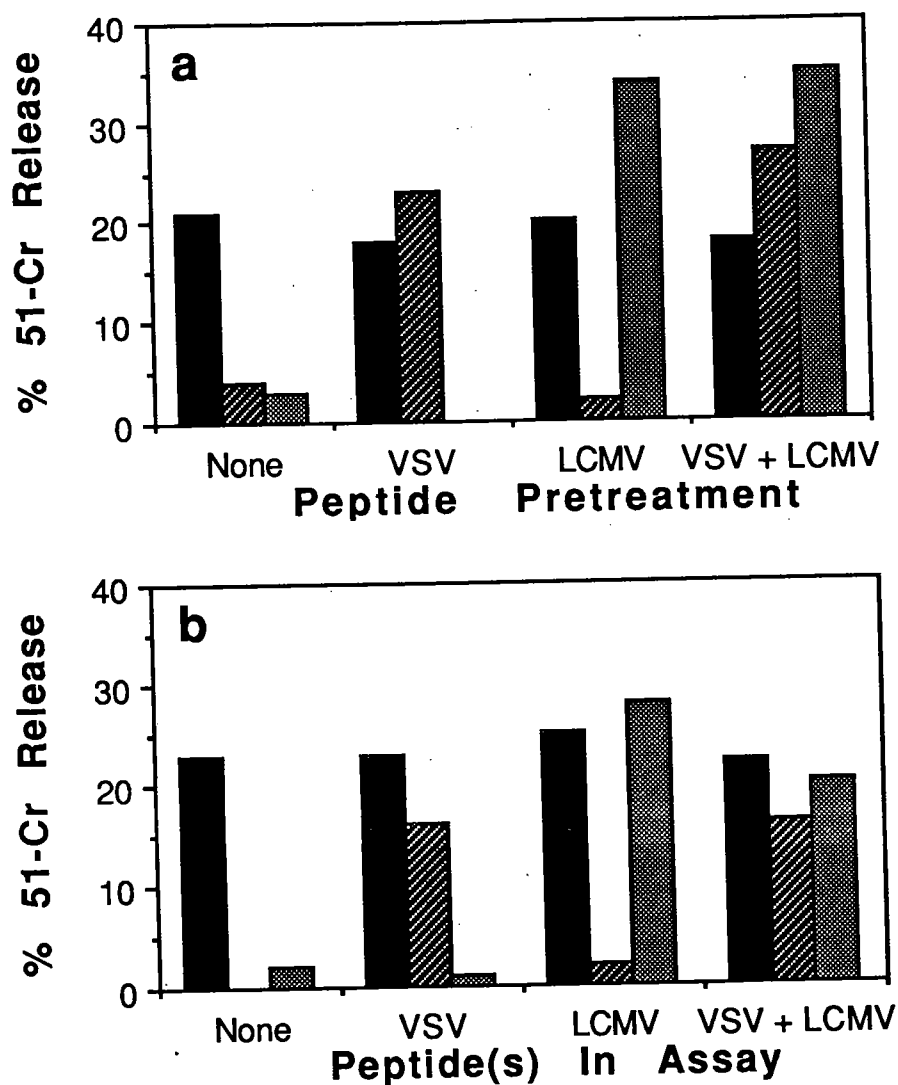
Figure 16. The combination of the VSV N and LCMV NP peptides added to P52 cells does not alter target cell susceptibility to NK cell-mediated lysis. ^{51}Cr-labelled P52 (H-2^b) targets were either preincubated overnight with the VSV N (K^b-restricted) and/or LCMV NP (D^b-restricted) peptides followed by washing (a), or the peptides were present throughout the assay at 10 μg/ml (b). Activated splenic NK cells (black bars), VSV-specific (striped bars) or LCMV-specific CTL (grey bars) were then added for a 6-h ^{51}Cr release assay. E:T ratio = 100:1.
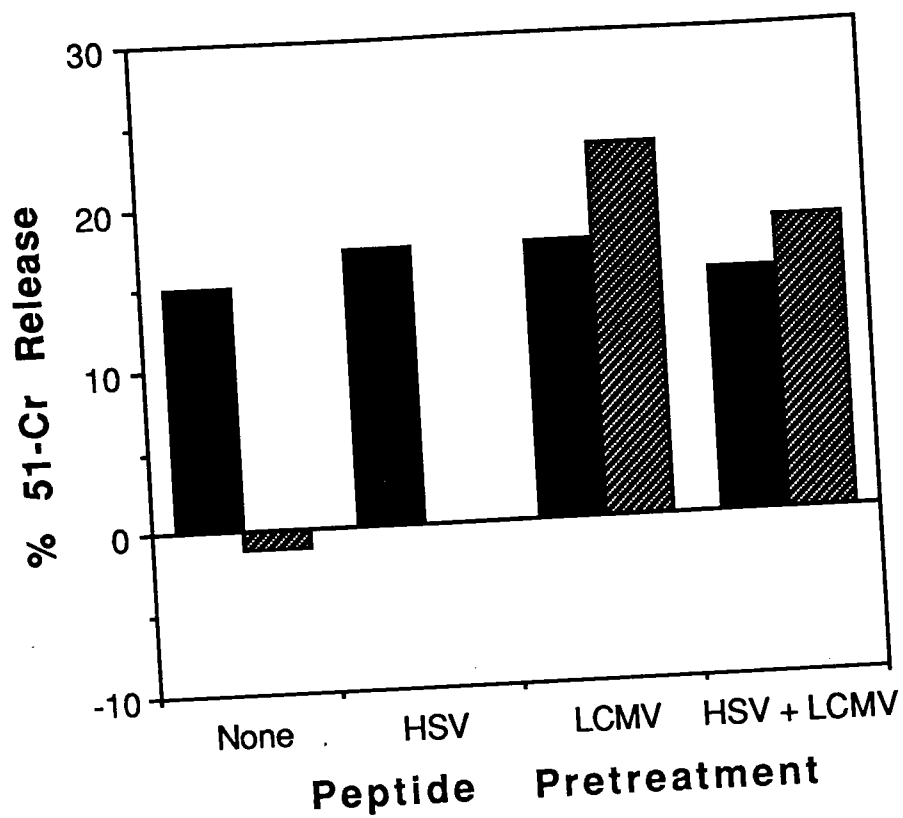
Figure 17. The combination of the HSV-1 gB and LCMV NP peptides added to P52 cells does not alter target cell susceptibility to NK cell-mediated lysis. P52 (H-2^b) targets were ^{51}Cr-labelled and preincubated overnight with the HSV-1 gB (K^b-restricted) and/or LCMV NP (D^b-restricted) peptides followed by washing. Activated splenic NK cells (black bars), or LCMV-specific CTL (day 10 post-infection, striped bars) were then added for a 5-h ^{51}Cr release assay. E:T ratio = 100:1.

CHAPTER VI

PATHOGENESIS OF MCMV INFECTION IN β_2-MICROGLOBULIN-DEFICIENT MICE

A. Replication of the Prototypic NK-Sensitive Virus, MCMV, in Intact or NK Cell-Depleted β_2-Microglobulin-Deficient or C57BL/6 Mice

MHC class I antigen expression has been implicated in the susceptibility of bone marrow grafts (Yu et al., 1992) or hematopoietic tumor cells (Höglund et al., 1991b) to control by NK cells *in vivo*. Bone marrow or tumor cells expressing allogeneic H-2 class I molecules are rejected by recipient NK cells, whereas those cells from syngeneic animals grow well *in vivo*. This is consistent with the "effector inhibition" model (Ljunggren and Kärre, 1990). β_2-microglobulin-deficient [β_2m (-/-)] mice have a defect in H-2 class I antigen expression (Koller et al., 1990; Zijlstra et al., 1990), and bone marrow cells from these mice are rejected in H-2 class I normal, heterozygous littermates (Bix et al., 1991), presumably due to the deficit in class I expression. In contrast, allogeneic H-2 class I+ bone marrow cells are able to grow in β_2m (-/-) recipients (Liao et al., 1991). These reports would seem to suggest that β_2m (-/-) mice would not have functional NK cells. However, NK cells from β_2m (-/-) mice are able to kill the mouse prototypic NK-sensitive target cell, YAC-1 (Höglund et al., 1991a; Liao et al., 1991), and therefore would be useful as an *in vivo* model in order to ask the question of whether H-2 class I molecule expression was required for the regulation of a

virus infection by NK cells. I infected intact or NK-depleted (injected with anti-asialo GM_1 antiserum) β_2-microglobulin-deficient [β_2m (-/-)] or C57BL/6 mice (both H-2^b) with the prototypic NK-sensitive virus, MCMV. MCMV is sensitive to NK cells *in vivo* (Bancroft et al., 1981; Bukowski et al., 1983, 1984, 1985) and is NK-sensitive independent of T or B cell functions (Welsh et al., 1991). The C57BL/6 mice served as an H-2 class I antigen (β_2m)-positive, MCMV-sensitive control. Three days post-infection, spleens were harvested, and virus was quantitated. The results presented are the average of 3 or 4 mice ± S.D. As shown in Table 7, splenic MCMV virus titers from C57BL/6 mice were approximately 0.5 - 1 $\log_{10}$ PFU higher in the NK-depleted group than NK-intact mice, as expected (Bukowski et al., 1983, 1984, 1985; Welsh et al., 1990; Shanley, 1990). Interestingly, a similar difference in MCMV titers was also observed in the spleens of NK-depleted β_2m (-/-) mice as compared to intact β_2m (-/-) mice. Decreases in splenic NK cell activity of anti-asialo GM_1-treated mice were observed, as measured by the lysis of YAC-1 target cells (Table 7). I do not know why the anti-asialo GM_1 treatment was more effective in the depletion of NK cell activity in C57BL/6 than in β_2m (-/-) mice. In our experience, the reduction in *in vitro* NK cell activity following anti-asialo GM_1 antiserum treatment of mice infected with the more cytopathic viruses, such as MCMV, is not as profound as with the noncytopathic virus, LCMV, or following injection with poly I:C. This will be addressed in more detail in the Discussion (Chapter VIII). Nonetheless, it is important

TABLE 7

Splenic MCMV Titers and NK Cell Activity in Intact and Anti-asialo GM1-Treated β2m (-/-) and C57BL/6 Mice[a]

Exp. #	anti-asialo GM1[b]	log10 PFU/spleen ± SD[c]		% YAC-1 Lysis[d]							
		β2m (-/-)	C57BL/6	β2m (-/-)				C57BL/6			
				100:1	50:1	25:1	12.5:1	100:1	50:1	25:1	12.5:1
1	-	<2.1 ± 0.1[e]	<2.0 ± 0.0[f]	27±3	23±4	18±5	14±4	19±2	16±2	12±5	7±2
	+	2.6 ± 0.1	<2.4 ± 0.4[e]	13±8	11±5	8±3	5±2	N.D.[g]	6±2	3±1	3±0.8
2	-	3.0 ± 0.3	3.3 ± 0.1	11±0	8±0.8	7±0.5	7±0.9	39±10	34±11	21±7	15±6
	+	4.6 ± 0.5	4.5 ± 0.2	11±1	8±1	6±2	5±3	4±1	4±0.8	4±1	3±0.8
3	-	3.3 ± 0.3	4.3 ± 0.1	37±2	31±3	21±2	12±0.5	51	27±9	19±5	13±4
	+	4.1 ± 0.2	4.4 ± 0.1	31±3	18±6	12±5	6±3	N.D.	N.D.	N.D.	7±0.8
4	-	3.4 ± 0.7	<2.5 ± 0.5[h]	N.D.	12±0	6±0.5	5±1	16±3	9±3	5±1	3±0.4
	+	3.8 ± 0.3	3.9 ± 0.2	N.D.	5±2	2±1	2±0.7	N.D.	1±0	1±1	1±1

[a] Age-matched β2m (-/-) and C57BL/6 mice were infected with 1 x10^5 PFU of MCMV, i.p.

[b] Mice received 20 μl of anti-asialo GM1 antiserum, i.p., or no treatment at the time of infection.

[c] Splenic MCMV titrated on C57BL/6 mouse embryo fibroblasts, 3 days post-infection.

[d] Splenic NK cell activity measured on day 3 post-MCMV infection at indicated E:T ratios.

[e] 1 of 3 mice below detection limit of assay.

[f] All mice below detection limit of assay.

[g] Not done.

[h] 2 of 4 mice below detection limit of assay.

to point out that the mice received the minimum effective dose of anti-asialo GM_1 antiserum, in order to avoid any nonspecific effects as can often occur when higher concentrations of anti-NK cell reagents are used *in vivo*, and that increases in splenic virus titers were observed in <u>both</u> groups of anti-asialo GM_1-treated mice.

B. Expression of H-2 Class I Antigens on Thymocytes Following MCMV Infection

During the course of a virus infection in normal mice, increases in the expression of H-2 class I molecules in various tissues is observed (Bukowski and Welsh, 1986a). In order to ensure that H-2 class I antigen expression in β_2m (-/-) mice was absent even after MCMV infection, so that the issue of whether or not H-2 class I molecules do play any role in the regulation of MCMV by NK cells could be addressed, thymocytes were isolated from both uninfected and MCMV-infected β_2m (-/-) and C57BL/6 (control) mice and stained for the expression of H-2D^b. Figure 18 shows that whereas increases in thymocyte expression of H-2D^b following MCMV infection of C57BL/6 mice can be detected by the antibodies, 28-11-5S and 28-14-8S, which recognize β_2m-associated and normal or free H-2D^b heavy chain, respectively (Townsend et al., 1989), no alterations in cell surface H-2D^b could be found in MCMV-infected β_2m (-/-) mice. Taking the above data together, the results suggest

that, unlike other systems (e.g., hematopoietic tumor cell rejection) in which a role for NK cells has been implicated, the regulation of a virus infection by NK cells, at least in the case of MCMV, is <u>independent</u> of MHC class I antigen expression.

C. *In vitro* and *in vivo* Evidence that IFN-Mediated Protection from NK Cell-Mediated Lysis Requires H-2 Class I Antigen Expression

As mentioned in Chapter I, IFN is produced early during a virus infection (Welsh, 1978) and has been shown to upregulate the expression of MHC class I antigens on various cells (Lindahl et al., 1976; Bukowski and Welsh, 1985, 1986a). This has been correlated with the ability of IFN to protect targets from lysis by NK cells (Trinchieri and Santoli, 1978; Welsh et al., 1981). Because thymocytes from uninfected, H-2 class I-normal mice are sensitive to lysis by NK cells, whereas those from virus-infected animals are resistant, thymocytes can be used to monitor IFN-mediated protection *in vivo*. Therefore, I compared the sensitivity of thymocytes from C57BL/6 and the H-2 class I-deficient, β_2m (-/-) mice to NK cell-mediated lysis before and after infection with MCMV. This would provide a more direct assessment of whether MHC class I molecule expression is necessary for IFN-mediated protection from NK cell-mediated lysis. I also analyzed the NK-sensitivity of normal and β_2m (-/-) cell lines before and after IFNβ treatment and compared this to the expression of H-2 class I antigens. In the

evaluation of the cell lines *in vitro* as shown in Table 8, only the β_2m-normal cells were able to be protected from NK cell-mediated lysis following IFNβ pretreatment; β_2m (-/-) cells were killed equally well with or without overnight exposure to IFNβ. This was also reflected *in vivo*. As Table 9 shows, thymocytes from uninfected C57BL/6 or β_2m (-/-) mice were very sensitive to lysis by LCMV-activated splenic NK cells from C3H/HeSnJ mice. Three days following MCMV infection, however, the C57BL/6 thymocytes were completely resistant to NK cell-mediated lysis. In contrast, this profound level of IFN protection observed with C57BL/6 mice was not demonstrated when thymocytes obtained from MCMV-infected β_2m (-/-) mice were used as targets. The moderate reduction in the sensitivity of thymocytes from MCMV-infected β_2m (-/-) mice to NK cell-mediated lysis, observed in Table 9, may have been due to differences in thymocyte subpopulations (as reflected in a preliminary experiment assessing CD4 and CD8 expression following MCMV infection) when compared to C57BL/6 mice (Figure 19; thymocytes were from Table 9, exp. 3). It should be noted that a cortisone-sensitive thymocyte subpopulation (presumably CD4+CD8+ double positive) appears to be the most sensitive to lysis by NK cells (Hansson et al., 1979), and this subpopulation is greatly reduced in the MCMV-infected β_2m (-/-) mice. Nonetheless, the most important conclusion from these experiments is that the degree of IFN-mediated protection was inversely correlated with H-2 class I antigen expression in both thymocytes (Figure 18) and cell lines
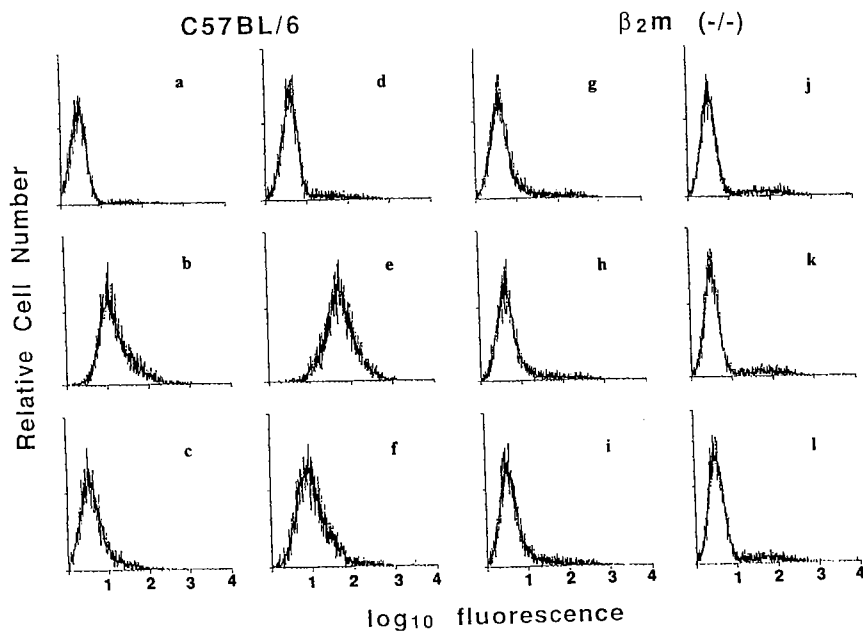
Figure 18. Expression of H-2D^b on thymocytes from uninfected or MCMV-infected C57BL/6 and β2m (-/-) mice. Single cell suspensions of thymocytes were prepared from uninfected or MCMV-infected (3 days post-infection) C57BL/6 or β2m (-/-) mice and were stained with the conformational-dependent, 28-11-5S (b, e, h, k) or conformational-independent, 28-14-8S (c, f, i, l), anti-H-2D^b monoclonal antibodies, followed by a FITC-conjugated goat anti-mouse Ig antiserum. Analysis was by FACStar. a-f, staining of H-2D^b on thymocytes from uninfected (a-c) or MCMV-infected (d-f) C57BL/6 mice. g-l, staining of H-2D^b on thymocytes from uninfected (g-i) or MCMV-infected (j-l) β2m (-/-) mice. Staining with second antibody only (a, d, g, j).

TABLE 8

Sensitivity of IFNβ-Treated Normal and β2m-Deficient Cell Lines to NK Cell-Mediated Lysis

Target Cells	Treatment[b]	% Lysis[c]	Mean Channel Fluorescence[a]			
			Control	M1	D[b]	D[b]/L[d]
R1.1 (H-2k)	Mock	42	4	44	N.D.	N.D.
	IFNβ	22	5	150	N.D.	N.D.
R1E (β2m-def.) (H-2k)	Mock	35	10	20	N.D.	N.D.
	IFNβ	34	5	13	N.D.	N.D.
D1R (H-2k + Db)	Mock	37	9	N.D.	266	163
	IFNβ	20	8	N.D.	447	247
R1E/Db (β2m-def.) (H-2k + Db)	Mock	36	20	N.D.	29	169
	IFNβ	36	12	N.D.	17	86

[a]Cells were stained with M1 (pan anti-mouse class I), 28-11-5S (anti-Db) or 28-14-8S (anti-Db/Ld) antibodies, followed by a FITC-conjugated F(ab)'2 goat anti-mouse Ig antiserum. Analysis was by FACS.

[b]Cells were either mock-treated or exposed to 1000 U/ml of murine IFNβ for 24 h at 37°C.

[c]Percent lysis by LCMV-activated splenic C3H/HeSnJ NK cells in a 4-h 51Cr release assay. E:T ratio = 100:1.

TABLE 9

Killing of Thymocytes from Uninfected or MCMV-Infected β2m (-/-) and C57BL/6 Mice by Activated NK Cells

| | % Lysis by C3H/HeSnJ NK cells[a] at E:T of: | | | | | |
| | Exp. 1 | | Exp. 2 | | Exp. 3 | |
Thymocytes from[b]:	50:1	12.5:1	50:1	12.5:1	50:1	12.5:1
Uninfected β2m (-/-)	22 ± 3	14 ± 2	39 ± 3	30 ± 2	37 ± 0.5	19 ± 4
β2m (-/-) + MCMV	15 ± 2	10 ± 2	18 ± 8	17 ± 4	16 ± 3	4 ± 2
Uninfected C57BL/6	17 ± 1	10 ± 0.5	27 ± 2	20 ± 1	30 ± 1	16 ± 2
C57BL/6 + MCMV	-4.2 ± 5.6	-1.3 ± 4.3	-0.7 ± 5	3 ± 2	-2.5 ± 2.5	-1.6 ± 4.1

[a]Spleen cells from C3H/HeSnJ mice, 3 days after LCMV infection, served as a source of activated NK cells. Lysis is presented as the average of 3 mice ± S.D.

[b]Thymocytes were obtained from uninfected C57BL/6 or β2m (-/-) mice, or 3 days following an i.p. infection with 1 x 10^5 PFU of MCMV, labelled with 51Cr and used as targets in a 4-h 51Cr release assay.
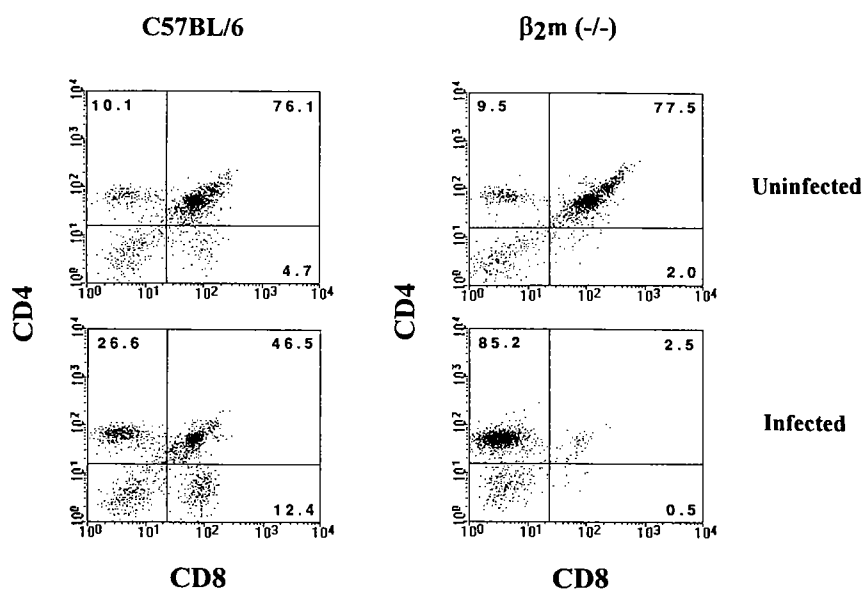
Figure 19. CD4 and CD8 expression on thymocytes from uninfected and MCMV-infected β_2m (-/-) and C57BL/6 mice. Single cell suspensions of thymocytes were prepared from individual uninfected or MCMV-infected (3 days post-infection) C57BL/6 and β_2m (-/-) mice and stained with PE-labelled anti-CD4 and FITC-conjugated anti-CD8 mAb. The presented profiles are representative of 3 or 4 mice in each group. The percentage of the CD4+CD8+ double positive and CD4+ and CD8+ single positive populations are indicated in the appropriate quadrants. The CD4-CD8- double negative populations in uninfected and infected groups ranged from 7 - 12% (C57BL/6) and 11 - 14% [β_2m (-/-)]. Two color analysis was by FACStar.

(Table 8). These results are in line with a previous report *in vitro* (Ljunggren et al., 1990) and are the first *in vivo* evidence suggesting that IFN-mediated protection <u>requires</u> the expression of MHC class I molecules, perhaps by delivering a negative signal to NK cells.

The above data strongly suggested that H-2 class I-dependent, IFN-mediated protection was not occurring in the β_2m (-/-) mice. I sought further evidence for this from another angle. If splenic NK cells were activated in the MCMV-infected β_2m (-/-) mice, and the non-NK cells in the spleen were not IFN-protected, there should be fewer spleen cells in NK-intact compared to NK-depleted (anti-asialo GM_1-treated) mice. When I analyzed the spleen cell number data from all 4 experiments presented in Table 7, I found that in MCMV-infected, anti-asialo GM_1-treated β_2m (-/-) mice, there were 1.4 ± 0.8 x 10^7 cells (n=13), whereas spleen cells from MCMV-infected, NK-intact β_2m (-/-) mice averaged 9.4 ± 5.6 x 10^6 (n=12; p<0.1). In MCMV-infected, normal C57BL/6 mice, the opposite result was observed (NK-intact: 1.3 ± 0.5 x 10^7; anti-asialo GM_1-treated: 7.0 ± 5.0 x 10^6 cells; n=13; p<0.01). These results are direct evidence for a lack of IFN-mediated protection in MCMV-infected β_2m (-/-) mice.

CHAPTER VII

IDENTIFICATION OF THE MOIETY RECOGNIZED BY mAb CZ-1

A. Characteristics of the CZ-1 Antigen

This laboratory has recently described the properties of a rat anti-mouse mAb designated CZ-1 (Vargas-Cortes et al., 1992). The CZ-1 mAb defines an epitope expressed on 15-25% of mouse bone marrow cells, 3-5% of thymocytes, and about 65% of splenocytes. The antigen is sensitive to treatment of cells with proteases and neuraminidase, but not to phosphatidylinositol (PI) phospholipase C, suggesting that it is associated with a non-PI-linked sialoglycoprotein. The molecule is expressed on B cells and virtually all NK cells and CD8+ T cells. However, it is not expressed on most peripheral CD4+ T cells unless they are first activated by T cell receptor stimulation. It is an early bone marrow marker for lymphocyte hematopoietic precursor cells, and it defines NK cells and their precursors at all detectable stages of their generation (Vargas-Cortes et al., 1991). Unfortunately, due to the low affinity of the CZ-1 mAb, previous efforts by others in the laboratory to identify the properties of the CZ-1 antigen by conventional biochemical analyses such as immunoprecipitation, Western blots and cross-linking methods were unsuccessful. FACS analyses, however, suggested that the CZ-1 antigen was a sialic acid-dependent epitope of a non-PI-linked membrane glycoprotein (Vargas-Cortes et al., 1992). The leukocyte staining pattern of mAb CZ-1 was not identical to any

other mAb that I have seen, but there are certain similarities in its staining distribution to the OX-22 anti-rat mAb (Table 10), which recognizes an isoform of CD45 (Spickett et al., 1983).

The leukocyte common antigen CD45 represents a ubiquitous group of membrane glycoproteins of 180-220 kD arising as a consequence of alternative splicing of at least 3 exons (exons 4, 5, and 6 or, A, B, and C; Figure 20) into 8 different cell surface isoforms (Johnson et al., 1989; Trowbridge, 1991). These proteins are heavily glycosylated, allowing for substantially increased heterogeneity due to post-translational modifications. CD45 molecules are transmembrane protein tyrosine phosphatases thought to be important for coupling T cell antigen receptor signals to the phosphoinositol second messenger system. Both CZ-1 and OX-22 identify hematopoietic precursor cells and stain a low percentage of thymocytes and a high percentage of splenocytes; some of these staining properties have also been seen with antibodies to various other isoforms of mouse CD45 (Bottomly et al., 1989; Hathcock et al., 1992). These characteristics led me to question whether mAb CZ-1 recognized a form of mouse CD45.

TABLE 10

Comparative Analysis of Cells Staining
with CZ-1 and OX-22[a]

	% OF CELLS STAINING WITH:	
CELL POPULATION	CZ-1	OX-22
B cells	100	100
CD4$^+$ T cells (naive)	6	50-75
CD8$^+$ T cells (")	100	100
NK cells	100	$\leq$100
Bone marrow cells	10-25	~50
Thymocytes	3-5	2

[a]Summary of data taken from Spickett et al., 1983; Vargas-Cortes et
al., 1992.
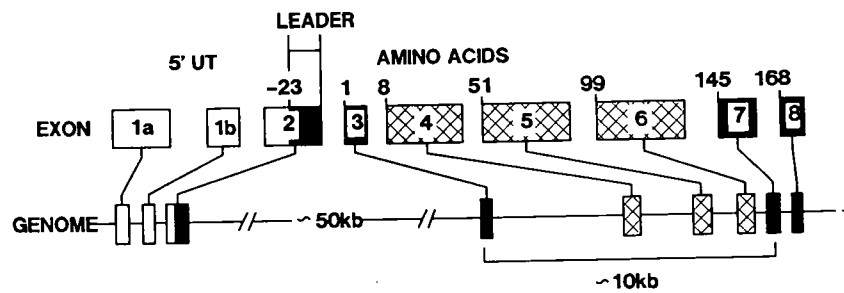
Figure 20. Genomic organization of the first 5' proximal 8 exons of the mouse CD45 gene. From Saga et al., 1990.

B.   Competitive Antibody-Binding Studies

Although the CZ-1 antigen is absent from many lymphocyte cell lines, we found both it and CD45 to be expressed on the pre-B cell line, 18.81.   The 18.81 cell line expresses several isoforms of CD45, but expresses in highest abundance the isoform containing the exons A, B, and C and in moderate abundance the isoform containing exons B and C (Saga et al., 1990).   Competitive binding studies were therefore performed on this line using double color FACS analyses and antibodies to several exons of CD45.   Cells were exposed simultaneously to mAb CZ-1 and to mAb panreactive with all mouse CD45 molecules or to exons A, B, or C.   As a control experiment, the rat anti-mouse IgM J11d was used in place of mAb CZ-1.   Figure 21 shows that, whereas none of the CD45-specific mAb inhibited the staining of cells with mAb J11d, the B-exon-specific mAb (CD45RB) caused a substantial inhibition in the binding of mAb to the cells (33% vs. 4% staining).   Antibody to exon C (CD45RC) also caused a moderate inhibition in staining with mAb CZ-1 (33% vs. 15%), but it should be noted that exon C is only expressed on this cell line in isoforms that also express exon B (Saga et al., 1990).   Figure 21 also shows a general correlation between how brightly mAb CZ-1 and the other CD45 mAb (CD45RA and CD45) stained 18.81 cells in combinations in which there was no competition.   Thus, the cells that expressed high levels of the CZ-1 antigen also expressed high levels of CD45.   It should be noted that B cells predominantly express a

form of CD45 which contains all three exons (Johnson et al., 1989; Saga et al., 1990; Trowbridge, 1991), but only antibody to the B exon caused an almost complete inhibition of mAb CZ-1 binding.

C. Expression of the CZ-1 Antigen on CD45-Transfected Cells

Ψ2 cells transfected with various isoforms of mouse CD45 (Johnson et al., 1989) were assessed for their ability to react with mAb CZ-1. Figure 22 shows that the nontransfected parent cell line did not react at all with mAb CZ-1 or the CD45 framework-specific mAb. Cells transfected with a CD45 gene without the alternatively spliced exons (T200/0) reacted with the CD45 framework-specific mAb but not at all with mAb CZ-1. Cells transfected with CD45-containing exon C (T200/C) reacted with C exon-specific mAb but not at all with mAb CZ-1. In contrast, cells transfected with CD45 + exons A, B, and C (T200/ABC) or CD45 + exons B and C (T200/BC) reacted with the appropriate anti-CD45 mAb and also reacted at lower but significant levels with mAb CZ-1. Although the level of staining with mAb CZ-1 was low, this was a reproducible positive result, highly consistent with the antibody blocking data shown in Figure 21. In these experiments, each Ψ2 transfectant cell line was reacted with all of the various anti-CD45 mAb in order to confirm expression of the appropriate exons, but Figure 22 for simplicity only shows data for the most meaningful mAb for each transfectant cell line.

One might argue that because the staining of the $\Psi 2$ transfectants with mAb CZ-1 was low, it might not necessarily be CD45RB-specific staining. To ensure that the mAb CZ-1 was indeed reacting with a CD45RB-associated epitope on the $\Psi 2$ transfectants, I performed a competitive FACS analysis as was presented in Figure 21. As Table 11 shows, mAb CZ-1 stained 11% and 71% of T200/ABC and 18.81 control cells, respectively, in the absence of any other mAb. When an antibody specific for CD45RB was added at the same time as mAb CZ-1, a significant inhibition in the staining with mAb CZ-1 was found (2% staining with T200/ABC; 11% with 18.81 cells). The addition of a CD45 framework-specific antibody with mAb CZ-1 to these cells did not inhibit mAb CZ-1 binding. Therefore, these results suggest that mAb CZ-1 recognizes a novel CD45RB-associated epitope.

D. Sialic Acid-Dependence of the CZ-1 Epitope

This laboratory had previously reported that treatment of spleen cells with neuraminidase, which cleaves sialic acid residues from the surface of cells, would abrogate the ability of the CZ-1 mAb to bind (Vargas-Cortes et al., 1992). This indicated that the expression of the CZ-1 epitope was sialic acid-dependent. In order to determine whether sialic acid was actually part of the CZ-1 epitope-binding site, binding competition studies were done on 18.81 cells between mAb CZ-1 and either sialic acid or the larger neuramin-

lactose. Figure 23 shows that 50 mM sialic acid caused a modest inhibition in the binding of mAb CZ-1 but not of mAb to H-2 class I molecules (M1) or mAb J11d. The larger neuramin-lactose molecule at 50 mM caused a very significant selective inhibition of binding by mAb CZ-1 but not by the two control antibodies. These results thus indicate that sialic acid is likely a part of the CZ-1 epitope. This could account for both its expression on a subpopulation of CD45 molecules as well as for its low affinity, as IgM antibodies to carbohydrates are often of low affinity.
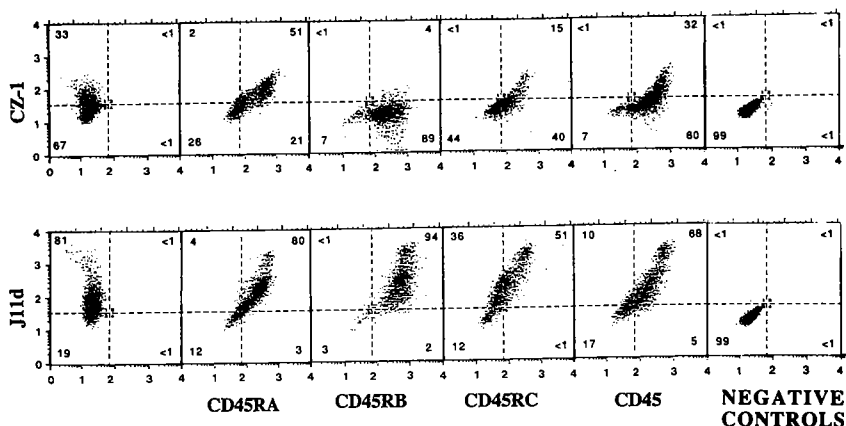
Figure 21. Competitive FACS analysis of mAb CZ-1 and CD45RA, RB, RC, and CD45 framework antibodies on 18.81 cells. The pre-B cell line, 18.81, was treated with mAb CZ-1 or, as a control, another rat anti-mouse IgM mAb that stains B cells, J11d. At the same time, cells were also exposed to the anti-CD45 mAb. The cells were then stained with a PE-goat anti-rat IgM (μ chain-specific) antiserum and a FITC-conjugated goat anti-rat IgG (γ chain-specific) antiserum. The $\log_{10}$ fluorescence of each color is indicated on the appropriate axis of the presented two-color profiles. Numbers in the quadrants represent the % of cells per quadrant. Negative controls: Top: FITC-conjugated goat anti-rat IgG only; Bottom: PE-goat anti-rat IgM only.
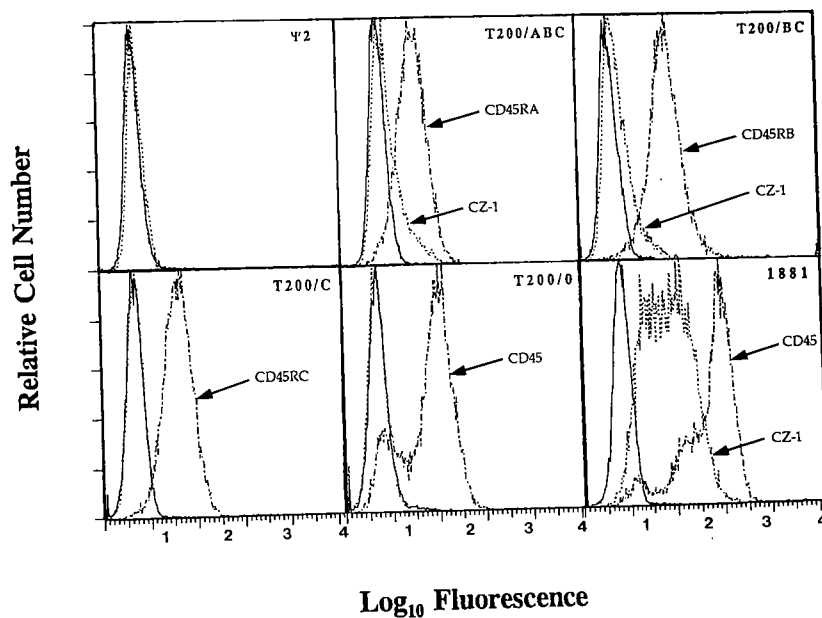
Figure 22. FACS analysis of Ψ2 cells transfected with various isoforms of CD45. The indicated Ψ2 transfectants, parental line and the CZ-1+ control pre-B cell line, 18.81, were stained with mAb CZ-1 or with mAb specific for the indicated CD45 exons, followed by a FITC-labelled goat anti-rat Ig antiserum. The parental cells were stained with mAb CZ-1 or with the CD45 framework antibody. The solid line in each profile shows the staining obtained with the FITC-conjugated second antibody alone. In three of the profiles (Ψ2, T200/C, T200/0) the staining with mAb CZ-1 directly overlapped the negative control.

TABLE 11

Antibodies to CD45RB (but not CD45 Framework)
Inhibit Binding of mAb CZ-1 to Ψ2/ABC and 18.81 Cells

Cell Line	% of Cells Expressing CZ-1[a]		
	CZ-1 Alone	CZ-1 + anti-CD45RB	CZ-1 + anti-CD45 framework
T200/ABC	11	2	15
18.81	71	16	77

[a]The indicated cell lines were stained with mAb CZ-1 and/or a biotinylated anti-CD45RB or anti-CD45 framework mAb, followed by SA-PE and a FITC-conjugated goat anti-rat Ig (μ-specific) antiserum. The percent CZ-1+ cells was determined by 2-color FACS analysis using a FACScan (Becton-Dickinson) program.
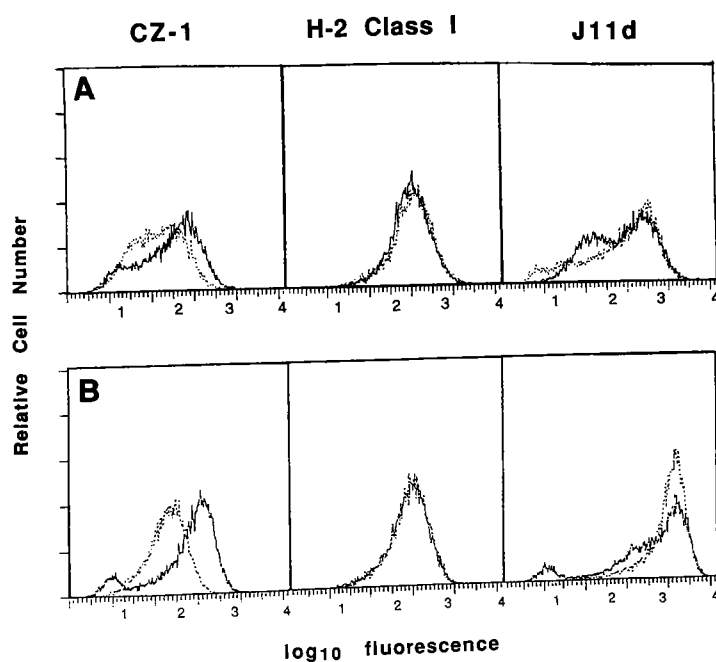
Figure 23. Inhibition of mAb CZ-1 binding to 18.81 cells by sialic acid and neuramin-lactose. 18.81 cells were incubated with (dotted lines) or without (solid lines) 50 mM sialic acid (A) or neuramin-lactose (B) in the presence of mAb CZ-1, anti-class I mAb, or J11d. Cells were then washed and stained with FITC-labelled goat anti-rat Ig. Analysis was by FACS.

CHAPTER VIII

DISCUSSION

The major focus of this thesis was to investigate the regulation of a virus infection by NK cells and its relationship to NK cell triggering. We first attempted to find conditions under which target cells became more sensitive to NK cell-mediated lysis and, following observations made during that study, to determine if the levels of H-2 class I molecules or if viral peptide charging of H-2 class I molecules played a role in the lysis of targets by NK cells. Furthermore, because of the animal models used, the more broad question concerning a role for MHC class I antigens themselves in the regulation of a virus infection by NK cells *in vivo* could also be asked.

The identity of the signal transduction molecule recognized by the novel mAb CZ-1, which was generated in this laboratory and reacts with all cytotoxic lymphocytes, including NK cells, was also investigated. Its identification, as well as the characteristics of those cells which express it, will help in understanding many of the effector cell-mediated phenomena which occur during the course of a virus infection.

VV-infected cells are sensitive to NK cell-mediated lysis during a "window of vulnerability". We initially set out to determine if target cells could be more sensitive to lysis by NK cells during a discrete period of time post-infection. This question was based on

previous observations showing that in overnight assays, virus-infected cells appeared to be more <u>sensitive</u> to NK cell-mediated lysis as compared to uninfected cells (Trinchieri and Santoli, 1978), whereas in short-term assays, virus-infected cells were often more <u>resistant</u> to lysis by NK cells (Welsh and Hallenbeck, 1980). Trinchieri and Santoli had demonstrated that in the overnight assays, virus-induced IFN activated the NK cells, augmenting the target cell lysis (Trinchieri and Santoli, 1978; Santoli et al., 1978b). Because in many previous studies by these investigators and others the target cells were infected for 1 to 3 days (depending upon the virus), it was conceivable that during a certain period of time post-infection, target cells could be more sensitive to lysis by NK cells and that the earlier investigations had assayed NK activity at times other than those of enhanced target cell susceptibility to NK cell-mediated lysis. In a time course study using short term (4 h) assays, we found that VV-infected L929 cells become more sensitive to lysis by NK cells during a discrete window of time, which occurred approximately 24 h post-infection and then subsequently declined (Figure 5). Therefore, this supported the hypothesis that the above studies might have indeed missed the time when virus-infected cells were more susceptible than uninfected cells to NK cell-mediated lysis, and is completely in line with a previous study demonstrating that target cells, when infected for longer periods, can become resistant to lysis by NK cells (Welsh and Hallenbeck, 1980).

A significant body of evidence has been presented in support of an inverse correlation between target cells expression of MHC class I antigens and susceptibility to NK cell-mediated lysis (Ljunggren and Kärre, 1990). Following VV infection and in line with previously published reports (Maudsley and Pound, 1991), it was found that the cell surface expression of H-2K^k and H-2D^k on L929 cells was decreased 2- and 5-fold, respectively, by 24 h post-infection (Figure 7), which corresponded to the "window of vulnerability". VV infection of cells results in the inhibition of host cell protein synthesis (Moss, 1968), and this is probably why we observed the quantitative decrease in H-2 class I expression. This quantitative 2- to 5-fold change in target cell H-2 class I antigens following VV infection, may have been sufficient to enhance target cell susceptibility to lysis by NK cells found during the "window of vulnerability". This is completely in line with the inverse correlation between MHC class I antigen expression and sensitivity to NK cell-mediated lysis.

It was also found that there was a decrease in the binding of effectors to target cells as the VV infection of the target cells progressed (Table 2). This suggests that for a given bound NK cell, its ability to lyse a target was significantly enhanced, perhaps due to increased stimulation of the NK cell. When the triggering of the NK cells upon interaction with VV-infected targets was investigated using a [$^{45}Ca^{2+}$] influx assay, an enhanced triggering during the "window of vulnerability" was observed (Figure 6).

Treatment of L929 cells with conventional protein synthesis inhibitors, such as cycloheximide, which also have the capacity to decrease H-2 class I expression (Reichner et al., 1988), not surprisingly enhance target cell sensitivity to lysis by NK cells (Kunkel and Welsh, 1981). In contrast, treatments with agents that increase MHC class I antigen expression, such as IFN (Lindahl et al., 1976), will make target cells more resistant to lysis by NK cells (Trinchieri and Santoli, 1978; Moore et al., 1980; Welsh and Hallenbeck, 1980; Welsh et al., 1981; Piontek et al., 1985). Using the [$^{45}Ca^{2+}$] influx assay, a correlation between the reduction in H-2 class I antigen expression and triggering of the effector cells when targets were treated with these reagents was also seen (Table 3). With regard to the experiment with cycloheximide, we believe that this is the first observation that cycloheximide treatment of target cells results in an increase of NK cell triggering. Overall, therefore, the ^{51}Cr release, H-2 class I antigen expression and triggering results during the "window of vulnerability" are consistent with the inverse correlation between MHC class I antigen expression and sensitivity of targets to NK cell-mediated lysis. The "window of vulnerability" may be the point of attack by NK cells *in vivo*, as VV has been reported to be an NK-sensitive virus (Bukowski et al., 1983, 1987).

Qualitative changes in H-2 class I molecules following V V infection suggested alterations in endogenous peptide presentation. Surprisingly, a nearly complete loss in the susceptibility of VV-

infected targets to lysis by allospecific CTL was observed at a time when the cells remained very sensitive to VV-specific CTL or NK cells. However, we had found what appeared to be only a moderate 2- to 5-fold decrease in H-2 class I expression following VV infection (Figure 7). Therefore, we had to be sure that this decrease in the level of H-2 class I molecules per se was not accounting for the abrogation of target cell sensitivity to lysis by allospecific CTL. Using L929 variants or cycloheximide-treated cells which expressed levels of H-2 class I antigens at or significantly below those observed following VV infection, it was found that these targets were still quite susceptible to allospecific CTL-mediated lysis (Figures 12, 13). These results, therefore, suggested that qualitative, rather than the quantitative changes in H-2 class I antigens, were important for this resistance to allospecific CTL-mediated lysis.

Allospecific CTL recognize endogenous peptides in the context of MHC class I molecules (Parham et al., 1987; Heath et al., 1989), and the level of available endogenous peptide antigen is a critical factor in determining whether targets are recognized and killed by CTL (Milligan et al., 1990). If the generation of endogenous peptides requires protein synthesis, then VV infection, which inhibits cellular protein synthesis (Moss, 1968), would decrease the concentration of these peptides available to newly synthesized MHC class I molecules. As a result, the susceptibility of L929 cells to killing by allospecific CTL would be predicted to decrease upon infection with VV, and this was observed (Figure 8). The approach taken to further investigate

this hypothesis involved the use of L929 cells, persistently-infected with LCMV [L(LCMV) cells] which, because they express endogenous LCMV peptides, are sensitive to lysis by LCMV-specific CTL. Upon superinfection with VV, L(LCMV) cells became resistant to lysis by allospecific and LCMV-specific CTL (Figure 14). Therefore, the loss in susceptibility of L(LCMV) cells to LCMV-specific CTL suggests that VV-encoded peptides successfully compete with LCMV (i.e., endogenous) peptides for newly synthesized H-2 class I molecules.

Viral peptide charging of H-2 class I molecules alters target cell sensitivity to NK cell-mediated lysis: a testable hypothesis. The results obtained with the allospecific CTL led us to question whether viral peptide charging was also responsible for the enhanced susceptibility of VV-infected cells to NK cell-mediated lysis. I should point out one caveat here: applying the interpretation of the allospecific CTL experiments to NK cells requires the assumption that NK cells recognize the peptide/MHC class I molecule complex in a manner similar to CTL. Evidence published by others is consistent with the possibility that the peptides presented by the MHC class I molecules might be critical in target cell susceptibility to NK cell-mediated lysis. The work of Dawson, Storkus and their colleagues (Storkus et al., 1989a, 1989b, 1991), which showed that a particular residue blocking access to the peptide binding groove in HLA-A2 played a role in conferring NK-resistance to cells expressing this MHC class I molecule, is quite in accord with the hypothesis that peptides

are important in target cell sensitivity to killing by NK cells. This suggested that NK cells could interact with the peptide/MHC class I complex. Additionally, a report using an HSV-1 infection model with human NK cells, presented evidence similar to that which we found with VV (Kaufman et al., 1992). In that study, C1R cells transfected with various HLA class I molecules were considerably less sensitive to NK cell-mediated lysis when compared to nontransfected controls. Upon HSV-1 infection, however, the transfectants became much more sensitive to lysis. Nontransfected C1R cells did not differ in their susceptibility to NK cell-mediated lysis after HSV-1 infection. Without a significant alteration in HLA class I antigen expression following HSV-1 infection, these authors concluded that a qualitative change in class I antigens (i.e., viral peptide charging) might be conferring sensitivity to NK cell-mediated lysis in the C1R HLA transfectants (Kaufman et al., 1992).

We took a direct approach to ask whether viral peptide charging of H-2 class I molecules affects the sensitivity of targets to NK cell-mediated lysis. *In vitro*, synthetic immunodominant viral peptides will associate with MHC class I molecules in the presence of an exogenous source of β_2-microglobulin (i.e., FBS; Townsend and Bodmer, 1989; Yewdell and Bennink, 1992) and targets so treated will be sensitized for killing by virus (or peptide)-specific CTL. Therefore, the target cells were either pretreated with peptide overnight, followed by washing, or the entire ^{51}Cr release assay was performed with peptide present. With neither of these approaches

was a significant modulation of target cell susceptibility to lysis by NK cells detected. It should be pointed out that, at least in the overnight pretreatment protocol, greater than 80 - 90% of the class I molecules should be charged with the added peptides (M. Trucco, personal communication) and during a virus infection of mouse cells, only about 0.1 - 5% (dependent on the virus) of the H-2 class I molecules are charged with the immunodominant peptides (J. Yewdell and J. Bennink, personal communication). We also considered the possibility that adding a single immunodominant viral peptide specific for a single H-2 class I allotype was insufficient, in the event that viral peptide charging of all of the H-2 class I molecules on the targets was necessary to observe a difference in the sensitivity of peptide-treated targets to NK cell-mediated lysis. Therefore, peptides specific for each of the K^b and D^b class I molecules were added to an $H-2^b$ (P52 lymphoma) target, using both peptide treatment protocols described above. Even under these conditions, no change in the cytolysis of targets by NK cells was observed. It has been previously reported that the addition of certain synthetic viral (or other class I-binding) peptides to target cells *in vitro* will result in an upregulation of those H-2 class I antigens for which the peptides are specific (Townsend et al., 1989; Lie et al., 1991), whereas with other peptides, this change does not occur (Lie et al., 1991). It could therefore be argued that peptide-induced alterations in the quantitative expression of target cell class I antigens might affect the sensitivity of target cells to lysis by NK

cells. For example, the NK-sensitive RMA-S cells, which express approximately 20% of the normal level of H-2 class I molecules do, following treatment with K^b- and/or D^b-specific peptides, upregulate their levels of cell surface class I antigens (Townsend et al., 1989). However, this does not result in a modulation of the susceptibility of these target cells to NK cell-mediated lysis (Franksson et al., 1991).

A major concern when peptide is present throughout the assay is the potential for effects upon the NK cells themselves. This cannot be ruled out in these types of experiments. In fact, extremely high concentrations of peptide (>200 μg/ml) in my analyses were inhibitory (Figure 15). Therefore, in most of my experiments, a group of target cells were included that were pretreated with peptide overnight, followed by washing, in order to avoid this possible variable. It should be noted that while these studies were underway, it was reported that the addition to Con A blasts of various synthetic viral immunodominant peptides defined for mouse CTL, allowed the targets to become more sensitive to lysis by NK (actually LAK) cells (Chadwick and Miller, 1992; Chadwick et al., 1992). Additionally, a study in a human system published a few months after these reports also presented evidence that a number of synthetic immunodominant viral peptides sensitized targets to be more susceptible to lysis by NK cells (Storkus et al., 1992). These authors used an overnight incubation of the targets with peptide followed by a wash step, as we did. I do not know why we could not observe an enhanced sensitivity of the targets to NK (or LAK) cell-

mediated lysis under the conditions we used or those recently reported. Even though a wide variety of target cells was used, including fibroblasts, mastocytomas, pre-B cells, thymomas, fibroblasts or thymomas transfected with various H-2 class I molecules, as well as Con A blasts, we were unable to find that any member of the panel of 12 synthetic viral peptides could increase target cell susceptibility to NK cell-mediated lysis. Furthermore, we used peptides specific for essentially all mouse H-2 class I allotypes of the H-2^b (K^b, D^b), H-2^d (K^d, D^d, L^d) and H-2^k (only K^k, not D^k) haplotypes. The variety of the target cell and peptide combinations, as well as the several experimental conditions used, would lead me to believe that if a difference in target cell sensitivity to NK cell-mediated lysis could be conferred by ·peptides, it should have been detectable in my assays. Furthermore, several other groups besides ours have been unable to find a difference in the sensitivity of mouse or human target cells to NK cell-mediated lysis following treatment with MHC class I-binding peptides (R. Kiessling, H. -G. Ljunggren, and M. Trucco, personal communications), and the group reporting on the human data apparently cannot reproduce its results (personal communication from the authors).

Considering the *in vitro* results with VV in our system and with HSV-1 in the human system (Kaufman et al., 1992), which suggest that viral peptide charging of MHC class I molecules might modulate the sensitivity of target cells to NK cell-mediated lysis, this indirect evidence would, on the surface at least, be difficult to reconcile with

the peptide experiments. However, these data could also be explained by mechanisms independent of the viral peptide charging of MHC class I antigens. With respect to the results with VV, and as pointed out above, the enhanced sensitivity of VV-infected cells to NK cell-mediated lysis during the "window of vulnerability" may have been due to a qualitative and/or quantitative change in cell surface H-2 class I molecules. As mentioned often throughout this thesis, an inverse correlation between target cell MHC class I antigen expression and susceptibility to lysis by NK cells has been observed in numerous systems (Ljunggren and Kärre, 1990). Therefore, the increased sensitivity of VV-infected cells to NK cell-mediated lysis could simply be due to the VV-induced reduction in H-2 class I antigen expression, resulting in enhanced NK cell triggering and lysis of VV-infected targets. Consistent with this are the reports and our results demonstrating that agents which upregulate (IFNβ) and downregulate (cycloheximide) MHC class I antigen expression, decrease and increase, respectively, target cell susceptibility to lysis by NK cells, and NK cell triggering. The results from Leibson's group with HSV-1 (Kaufman et al., 1992), can also be interpreted by a mechanism that is independent of the charging of HLA class I molecules with viral peptides. In their report, these authors used C1R cells--a very NK-sensitive target. Because of this high NK-sensitivity, the killing of uninfected C1R targets by NK cells would be at a maximum. Infection of C1R cells with HSV-1 would not be able to exceed this maximal level of NK-sensitivity. On the other hand,

C1R cells, which are transfected with HLA class I molecules, are relatively resistant (or considerably less sensitive) to NK cell-mediated lysis, as has been previously demonstrated (Storkus et al., 1989a, b). The obvious correlation is with MHC class I expression. It has also been previously shown that tumor necrosis factor-α (TNFα), which is also produced by NK cells, will lyse cells infected with VSV, human CMV, Theiler's murine encephalomyelitis virus (TMEV), or HSV-1, at a higher level than that observed with uninfected cells (Paya et al., 1988). Furthermore, cells treated with protein synthesis inhibitors, such as actinomycin D or cycloheximide, are more sensitive to NK cell-mediated lysis (Kunkel and Welsh, 1981). Like other cytopathic viruses, HSV-1 has the capacity to inhibit host cell protein synthesis. It is possible that what appears to be a qualitative change in class I antigens being responsible for the increased lysis of HSV-1-infected C1R transfectants by NK cells is actually an enhanced sensitivity of the infected cells to the cytotoxic moieties produced by NK cells. This may have also played a role in the observation with VV-infected cells and will be discussed further in a later section.

I cannot formally rule out the possibility that viral peptide charging of MHC class I antigens can, at least in some cases, modulate target cell susceptibility to NK cell-mediated lysis. Rather than 1 or 2 different peptides being able to make a difference, it might require a particular heterogeneous set of peptides which would only be presented by MHC class I molecules during a virus infection. These peptides might then be sufficient to alter or prevent the proposed

negative signal delivered by the target cell class I molecules to the effector cells (Karlhofer et al., 1992; Ciccone et al., 1992). The addition of the natural peptides extracted from the NK-resistant RMA cell line to the NK-sensitive RMA derivative, RMA-S, has been reported to make the latter NK-resistant (Correa et al., 1992). Unfortunately, these authors did not indicate if the level of H-2 class I expression was affected by this treatment, as peptides added to these two cell lines will result in an increase in class I antigens on the cell surface (Townsend et al., 1989). *In vitro* results, however, are sometimes difficult to apply to that which occurs *in vivo*. Indeed, the one report suggesting that peptides can modulate target cell sensitivity to lysis by human NK cells did propose that "exogenous peptide effects on target cell sensitivity to NK are principally relevant *in vitro* and not *in vivo*", presumably due to the requirements for high concentrations of exogenous β_2m during peptide exchange at the cell surface (Storkus et al., 1992). Therefore, at this stage, it would be difficult to make a generalized statement on whether or not viral peptide charging of MHC class I molecules plays a role in the control of a virus infection by NK cells.

Are H-2 class I molecules themselves important in the regulation of a virus infection by NK cells *in vivo*? In order to ask the question of whether H-2 class I molecules were required for the regulation of a virus infection *in vivo*, NK-depleted or intact β_2m(-/-) mice were infected with the prototypic NK-sensitive virus, MCMV,

and then compared to the β_2m-normal, MCMV-susceptible, C57BL/6 mice (both groups of mice are H-2^b). As mentioned above, β_2m (-/-) mice are defective in the expression of H-2 class I molecules (Koller et al., 1990; Zijlstra et al., 1990). Therefore, these mice allowed us to directly assess the role of MHC class I antigens in the regulation of a virus infection by NK cells *in vivo*. In the C57BL/6 control mice, it was observed that splenic MCMV titers were much higher (~0.5 - >1 $\log_{10}$ PFU) in the NK-depleted group than in the NK-intact group (Table 7), as has been previously reported (Bukowski et al., 1983, 1984, 1985; Welsh et al., 1990; Shanley et al., 1990). Interestingly, in β_2m (-/-) mice, we also found a similar increase in splenic MCMV titers in anti-asialo GM$_1$-treated as compared to untreated mice. A difficult to explain observation was the fact that the anti-asialo GM$_1$ treatment depleted only about 50% of the splenic NK cell activity in the β_2m (-/-) mice, even though it was able to abrogate most of the NK cell activity in the C57BL/6 mice. I cannot explain why the treatment of the β_2m (-/-) mice with the anti-asialo GM$_1$ antiserum did not result in a more effective depletion of NK cell activity. Clearly, we found increases in splenic MCMV viral titers in anti-asialo GM$_1$-treated as compared to untreated mice, in both C57BL/6 and β_2m (-/-) mice. We were concerned about using higher concentrations of anti-asialo GM$_1$ antiserum because, although treatment of mice with this antiserum is rather selective for NK cells, higher doses can react with other cells, and this would cause problems in interpretation. The concentration of anti-asialo GM$_1$

antiserum that is used in our laboratory has been titrated on the basis of its ability to totally deplete LCMV-induced splenic NK cell activity. Cytopathic viruses, such as MCMV, which alter cellular function much more so than the less pathogenic LCMV, might be able to overcome the total NK depletion, perhaps by more quickly activating NK cells, as it takes about 12 - 24 h for the antiserum to ablate NK cell activity completely (Habu et al., 1981). It should be noted that associated with the activation of NK cells is an increase in the expression of glycosphingolipids, such as asialo GM_1 (Welsh, 1986). This problem has recently been observed on occasion by other members in the laboratory with MCMV, using both normal and SCID C57BL/6 mice, and we at present cannot explain the lower efficiency of this antiserum in mice infected with this virus. It is possible that if only 90% of the splenic NK cells were depleted by the anti-asialo GM_1 treatment, for example, the highly activated NK cells generated by signals stimulated by enhanced MCMV replication could account for the observed activity when compared to mice not depleted of NK cells and producing lower levels of activating factors. Also, with few CD8+ cells in the spleens of $\beta_2 m$ (-/-) mice (almost none), it would also make sense that the concentration of NK cells/total number of spleen cells would be higher than in normal mice. This would make a difference in the NK cell to target cell ratio and hence, be reflected in YAC-1 lysis. Nonetheless, the most important conclusion from the experiments presented in Table 7 is that the enhanced replication of MCMV in the spleens of anti-asialo

GM_1-treated β_2m (-/-) and C57BL/6 mice suggests that H-2 class I molecules <u>do</u> <u>not</u> play a role in the regulation of MCMV by NK cells.

Other systems in which NK cells have been shown to play a role have demonstrated that MHC class I molecules are particularly important. For example, the growth of transplanted hematopoietic tumor cells in mice is impaired unless the tumor recipient mice are completely MHC-matched with the donor tumor (Höglund et al., 1991b). Elimination of MHC-disparate tumors in this model is mediated by NK cells. The rejection of bone marrow cells in mice has also been shown to be somewhat dependent upon the MHC class I allotypes expressed (Yu et al., 1992). However, as described above, when the H-2 class I-deficient β_2m (-/-) mice were injected with MCMV and anti-asialo GM_1 antiserum, there were elevated viral titers as compared to mice not receiving the antiserum (Table 7). This is exactly what one observes with normal mice. If we also take into account the data presented in Figure 18, which shows that thymocytes from β_2m (-/-) mice do not express detectable levels of H-2D^b on their surface, even after MCMV infection, in contrast to the other models mentioned above, our evidence suggests that the regulation of MCMV by NK cells *in vivo* is <u>independent</u> of H-2 class I antigen expression.

Thymocytes obtained from uninfected normal mice express very low levels of MHC class I antigens, whereas following a virus infection, there is a profound increase in class I expression (Bukowski and Welsh, 1986a; Figure 18). In contrast, there is a lack

of detectable cell surface H-2D^b on thymocytes from uninfected or MCMV-infected β_2m (-/-) mice (Figure 18). The upregulation of MHC class I antigens on thymocytes during a virus infection is correlated with the production of IFN and with resistance to lysis by NK cells (Hansson et al., 1980; Bukowski and Welsh, 1986a). The experiments presented using both normal and β_2m (-/-) thymocytes (Table 9) and β_2m (-/-) and normal cell lines (Table 8), as well as a previously published report (Ljunggren et al., 1990), also support this correlation. In fact, the experiments with the β_2m (-/-) mice are the first *in vivo* evidence supporting a <u>requirement</u> for MHC class I antigen expression in IFN-mediated protection from NK cell-mediated lysis, and are consistent with the "effector inhibition" model (Ljunggren and Kärre, 1990). It has been previously shown that the ability of IFN to protect target cells from lysis by NK cells *in vitro* can be abrogated by viruses which can inhibit host cell RNA or protein synthesis (Trinchieri and Santoli et al., 1978; Bukowski and Welsh, 1985). Therefore, Trinchieri and Santoli proposed that *in vivo*, NK cells could selectively kill virus-infected cells, due to the fact that IFN cannot protect those targets from lysis by NK cells. Uninfected cells would be protected from both infection as well as NK cell-mediated lysis due to the effects of IFN (Santoli et al., 1978b). *In vitro* studies supported this concept with MCMV, which rendered mouse fibroblasts resistant to IFN-mediated protection (Bukowski and Welsh, 1985). In β_2m (-/-) cells, IFN treatment does not cause these cells to become resistant to lysis by NK cells, due to the lack of

H-2 class I upregulation (Ljunggren et al., 1990; Table 8). Therefore, *in vivo*, what could be occurring in these mice? IFN has at least four major roles in a virus infection: 1. activate NK cells; 2. induce an antiviral state in the uninfected cells; 3. protect uninfected cells from lysis by NK cells, presumably by upregulating the expression of MHC class I antigens; 4. sensitize targets for attack by CTL by upregulating class I molecules. In β_2m (-/-) mice, only 1 and 2 could be operative. However, the same apparent NK-sensitivity of MCMV occurs in these mice as in normal mice, as measured by splenic virus titers.

The above results do not necessarily mean that the lack of IFN protection is not playing some role during MCMV infection. In the *in vivo* experiments, it was always found that MCMV-infected, NK-depleted normal (C57BL/6) mice were much more sick (e.g., ruffled hair, hunched over back) than NK-intact animals. This should not be too surprising, as MCMV is an NK-sensitive virus and replicates to much higher titers in an NK-depleted host. However, NK-intact, MCMV-infected β_2m (-/-) mice were as sick or more so than similar mice which were also treated with anti-asialo GM_1 antiserum. Furthermore, the number of spleen cells from NK-intact β_2m (-/-) mice were <u>lower</u> than anti-asialo GM_1-treated mice following MCMV infection. This was the complete opposite of that which occurred in β_2m-normal C57BL/6 mice. Without the ability of IFN to protect uninfected cells in the β_2m (-/-) mice from attack by MCMV-induced, activated NK cells by upregulating MHC class I antigens (Figure 18),

this result makes sense. Therefore, although H-2 class I molecules do not appear to play a role in whether or not MCMV is NK-sensitive, the presence of these antigens may make a considerable difference in the resultant morbidity following infection. This would appear to be consistent with the hypothesis presented by Trinchieri and Santoli (Santoli et al., 1978b) and seems to warrant further investigation.

Possible MHC class I-independent mechanisms governing the regulation of virus infections by NK cells.

I have been discussing the relevance of my observations as they pertain to MHC class I expression. How might NK cells be able to kill MCMV (or other virus)-infected cells independent of H-2 class I expression? Essentially all viruses which have been shown, under at least some *in vitro* or *in vivo* conditions to be NK-sensitive, are cytopathic to the cells they infect. Therefore, rather than the virus infection modulating a target cell surface structure(s) which is inducing a negative signal (i.e., MHC class I molecules), these cytopathic viruses might cause targets to be more sensitive than uninfected cells to lysis by the cytotoxic moieties produced by NK cells. This would be a direct result of the cytopathic effect of the virus infection itself. For example, I mentioned that VSV-, CMV-, TMEV- and HSV-1-infected cells are more sensitive to NK cell-produced TNFα than uninfected cells *in vitro* (Paya et al., 1988). This could also have relevance *in vivo*, as a recombinant VV expressing TNFα is cleared in immunocompromised mice, whereas similar mice receiving the wild

type VV control virus succumb to the infection (Sambhi et al., 1991). The mechanism of this is unclear, but it has also been shown that a recombinant VV expressing IL-2 is eliminated in athymic nude mice (Flexner et al., 1987; Ramshaw et al., 1987). This effect is concomitant with the activation of NK cells (Karupiah et al., 1990a) and is dependent upon the production of IFNγ (Karupiah et al., 1990b), which can synergize with TNFα to mediate a number of effects upon cells in addition to antiviral activity (Wong and Goeddel, 1986). The antiviral properties of IFNγ itself cannot be ignored, as NK cells are the major producers of IFNγ early during a virus infection (McFarland and Bigley, 1990). The enhanced sensitivity of tumor cells to lysis by C' (Schlager and Ohanian, 1980) or NK cells (Kunkel and Welsh, 1981; Figure 13) following treatment with metabolic inhibitors, might imply an increased susceptibility of target cells to lysis by NK cell-produced perforin, which is homologous to C' component C9 (Tschopp et al., 1986; Young et al., 1986b; Zalman et al., 1986), following infection with a cytopathic virus. Therefore, there are also MHC class I-independent factors which, following a virus infection, could make target cells more sensitive to lysis by NK cells, in addition to affecting the growth of the virus. Any, or all, of these elements could be operative during a virus infection.

Effects of a virus infection on cytotoxic effector cells and possible role for the CZ-1 antigen. During the early phase of a virus infection, LGL NK cells accumulate at sites of virus replication (Welsh, 1986). For example, an i.p. infection with LCMV results in an increase in activated peritoneal NK cells (Welsh, 1978), whereas an intracerebral injection of mice with VV results in an increase of cells with NK-like activity in the cerebral spinal fluid (Doherty and Korngold, 1983). The chemotaxis of NK cells appears to correlate with the production of various cytokines produced during a viral infection--particularly, IFNβ and IL-2. For example, although IFNβ plays an important role in NK activation and proliferation, it can also act as a chemotactic agent for NK or LAK cells (Welsh and Vargas-Cortes, 1992). However, a number of other properties of IFNs can affect certain cell surface molecules which are involved in NK cell effector functions (Welsh, 1986). The Fcγ receptor (CD16) on NK and other cells is upregulated, which would be of significant importance for ADCC (Kiessling et al., 1980). Virus-induced IFNα/β does increase the expression of various sialoglycoproteins (Yogeeswaran et al., 1982), which may provide a means for activated NK cells to remain at sites of virus infection, as many sialoglycoproteins can bind lectins, and activated NK cells are somewhat more adherent to nylon wool than nonactivated (endogenous) NK cells. The effects of IFNs on the expression of the putative murine NK cell receptors, NKR-P1 and Ly-49, have not yet been reported. Although IL-2 can also activate NK cells and enhance their cytolytic effector capacity, unlike IFNβ, high

doses of IL-2 can stimulate *in vitro* NK cell proliferation, which generates the so-called LAK cells (Grimm et al., 1982). IL-2 can also serve as a chemotactic agent for virus-induced NK cells (Welsh and Vargas-Cortes, 1992). Whether or not IL-2 serves as a growth factor for NK cells *in vivo* is unclear, as IL-2 production parallels the CTL response and nude or SCID mice, which produce little if any IL-2, can generate activated NK cells (Welsh and Vargas-Cortes, 1992). However, injection of mice with IL-2 does induce NK cell proliferation *in vivo* (Biron et al., 1990), perhaps through the stimulation of IFNγ production, which also can cause NK cell proliferation when administered *in vivo* (Biron et al., 1984). However, considering the redundancy of the immune system, one cannot rule out a role for IL-2.

There may be some interdependence of IFNα/β and IL-2 during a virus infection. The cell surface expression of the signal transduction molecule, CD45, which is found on NK cells (as well as other leukocytes), can be upregulated by virus-induced IFNα/β (Welsh, 1986). The increase in CD45 expression appears to be a direct result of cell activation, and CD45 is important in T cell responses to anti-CD3 and IL-2 (Gilliland et al., 1990; Trowbridge, 1991). Additionally, certain anti-CD45 mAb can inhibit NK cell-mediated lysis (Seaman et al., 1981; Newman et al., 1983). It has been shown in this thesis that mAb CZ-1 recognizes a sialic acid-dependent epitope associated with a subpopulation of CD45RB molecules (Figures 21-23). Previous work in our laboratory has

shown that the CZ-1 antigen is expressed on all NK cells and CD8$^+$ T cells, but not on CD4$^+$ T cells or splenic macrophages (Vargas-Cortes et al., 1992). A paper in press from our laboratory (Copyright Notice, Brutkiewicz et al., 1993; also, Vargas-Cortes et al., 1991), has further demonstrated that only CZ-1$^+$ spleen cells or thymocytes can respond to IL-2. Splenic CD8$^+$ T cells, but not most CD4$^+$ T cells, proliferated in response to IL-2. This also helps explain why LAK cells, which are generated by incubating spleen cells or peripheral blood lymphocytes in high concentrations of IL-2, are devoid of CD4$^+$ T cells. Furthermore, sorting of spleen cells into CD45RB$^+$CZ-1$^+$ and CD45RB$^+$CZ-1$^-$ populations resulted in only the former population proliferating in response to IL-2. NK cells, which are all CZ-1$^+$, can be stimulated to higher levels of cytotoxicity following incubation with either IL-2 or IFNβ. The ability of mAb CZ-1 to block the NK cell activation induced by either of these cytokines (Vargas-Cortes et al., 1991) suggests the existence of a common pathway for the stimulation of enhanced NK cell-mediated cytotoxicity by these agents.

It is likely that the CZ-1 epitope is an activation/differentiation antigen which is selectively expressed on certain lymphoid subpopulations during various stages of ontogeny and, upon receiving the appropriate stimulus, can be upregulated in CZ-1$^-$ cells. For example, although resting CD4$^+$ T cells are CZ-1$^-$, following activation by a virus infection *in vivo*, or by anti-CD3 and IL-2 *in vitro*, there is a marked increase in CZ-1$^+$ cells in the splenic CD4$^+$

population (Vargas-Cortes et al., 1992). Other mAb specific for different epitopes of CD45RB have been used to distinguish between Th1 and Th2 (Bottomly et al., 1989) and virgin from primed/memory (Lee et al., 1990) CD4+ T cells. The CZ-1 mAb has been found to stain Th1 and Th2 cell lines in a manner similar to these mAb (P. Rogers and D. Parker, personal communication).

CD45 is a highly glycosylated molecule, and post-translational modifications might be predicted to have significant effects upon cells expressing various isoforms of CD45. There is evidence to suggest that the carbohydrate moieties expressed by CD45 molecules play an important role in T cell ontogeny and CD45 function. Thymic subpopulations differ in their expression of different carbohydrate epitopes on mouse CD45 molecules (Lefrancois, 1987). As mentioned above, our laboratory has previously shown that the CZ-1 antigen is also found in the thymus (Vargas-Cortes et al., 1992) and its expression is inducible in CZ-1- thymocytes (M. Vargas-Cortes and R. Welsh, unpublished observations). Recent evidence has demonstrated that blocking the glycosylation of CD45 by treatment of K562 or Daudi cells with tunicamycin, a specific inhibitor of N-linked glycosylation, will inhibit the cell surface expression of CD45RB (Pulido and Sánchez-Madrid, 1990; 1992), as well as the tyrosine phosphatase activity of CD45, further illustrating the importance of glycosylation in the expression and function of CD45 and CD45RB, in particular. The identification of the significance of the CZ-1 antigen (as well as other carbohydrate moieties on CD45

molecules) in these and other functions would therefore be of interest.

During the course of a virus infection, expression of the CZ-1 antigen could be very important in the late early stage, by playing a role in the accumulation of LGL at the site of infection perhaps due, at least in part, to the requirement of CZ-1 expression for IL-2 responsiveness. Furthermore, CZ-1+ CD4+ T cells may be major players in mediating the Th2 helper function for antibody production, as well as other activities; it appears that CD8+ CTL precursors do not need CD4+ Th cells for their generation into mature CTL, provided that there is sufficient IL-2 present (Rahemtulla et al., 1991).

The results described in this thesis have given us a clearer understanding of viral pathogenesis and of signal transduction in *in vitro* and *in vivo* NK cell activity. A number of important observations were made in this regard and are listed below:

1. VV-infected target cells are more sensitive to NK cell-mediated lysis during a "window of vulnerability".

2. This "window of vulnerability" is correlated with enhanced triggering of NK cells, but not with increased binding of NK cells to VV-infected targets.

3. Quantitative decreases in target cell H-2 class I molecule expression occur following infection with VV.

4. VV-infected target cells are resistant to lysis by allospecific CTL at a time when they are highly sensitive to lysis by VV-specific CTL, suggesting alterations in endogenous peptide presentation.

5. Viral peptide charging of H-2 class I molecules does not modulate target cell sensitivity to NK cell-mediated lysis.

6. *In vivo*, NK cells can regulate the prototypic NK-sensitive virus, MCMV, independent of H-2 class I antigen expression.

7. H-2 class I molecules are required for IFN-mediated protection from NK cells *in vitro* and *in vivo*.

The identification of the CZ-1 epitope as part of, or associated with CD45RB, raises new questions regarding signal transduction events which occur at the effector cell level, as well as during lymphocyte development. Therefore, this dissertation should provide a foundation for further studies of the immune response to virus infections.

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