THE IMMUNOBIOLOGY OF INFECTION WITH RECOMBINANT VACCINIA VIRUS ENCODING MURINE IL-2

by

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STATEMENT

With the exception of the following, the experiments described in this thesis represent my own work. The *in situ* mouse liver perfusions described in Chapter 4 were done in collaboration with Dr. Nick J. C. King. Enzyme-linked immunosorbent assays for murine interferon gamma described in Chapter 5 were performed by Dr. Janet Ruby.

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Antiviral, cytotoxic T (Tc) cells are essential for recovery from primary poxvirus infections. A recombinant vaccinia virus encoding murine IL-2 (VV-HA-IL2) was previously constructed by others who showed that athymic, nude mice, which lack functional T cells, resolved an infection with this virus whereas nude mice infected with a control virus not encoding IL-2 (VV-HA-TK) developed a disseminated vaccinial disease and died. The experiments described in this thesis were designed to elucidate immune mechanism(s) which contributed to the recovery of nude mice from VV-HA-IL2 infection.

Investigations into the kinetics of virus replication and clearance in nude and normal mice indicated that VV-HA-IL2 was rapidly cleared compared to VV-HA-TK, suggesting that the mechanism(s) involved in clearance of VV-HA-IL2 were induced rapidly within 1-4 days after infection.

Since IL-2 activates and induces the proliferation of T cells and natural killer (NK) cells, studies were undertaken to determine if these effector populations were involved in the process of rapid viral clearance.

Tc cell responses could not be detected in nude mice infected with either VV-HA-IL2 or VV-HA-TK. The magnitude and kinetics of appearance of Tc cell responses in normal CBA/H mice inoculated with VV-HA-IL2 or VV-HA-TK were similar. These findings excluded a role for Tc cells in the rapid elimination of VV-HA-IL2. However, splenocytes from nude and normal mice infected with VV-HA-IL2 had elevated NK cell activity which was 3-fold higher than the activity induced by VV-HA-TK. The VV-HA-IL2-induced elevated NK cell activity persisted in nude mice for up to 8 days and in normal mice for up to 3 days after infection. Effectors mediating lysis of YAC-1 targets were mainly as-GM1$^+$, Thy1.2$^\pm$, CD4$^-$ and CD8$^-$.

Experiments using mice depleted of NK cells with specific antiserum and beige mutant mice deficient in cytolytic NK cells provided evidence for a causal relationship between VV-HA-IL2-induced NK cells and their role in viral clearance. Depletion of NK cells in nude or normal mice greatly exacerbated VV-HA-IL2 infection and resulted in significant increases in viral titres.

A comparison of the kinetics of virus replication in CBA beige mice and normal CBA/H mice indicated that VV-HA-IL2 grew to higher titres and that virus clearance was delayed in beige mice compared to normal mice. VV-HA-IL2 induced strong splenic and ovarian NK cell activity which coincided with the rapid clearance of virus from ovaries of normal CBA/H mice; CBA beige mice had no detectable NK cell activity in spleens or ovaries. Nevertheless, VV-HA-IL2 was cleared more efficiently than VV-HA-TK in beige mice suggesting that mechanism(s) other than NK cell-mediated cytotoxicity were involved in rapid clearance of VV-HA-IL2.

The role of host derived cytokine(s) involved in the IL-2-induced mechanism(s) of antiviral activity was investigated. Administration of a monoclonal antibody (Mab) to IFN-γ inhibited the ability of nude mice to clear VV-HA-IL2 and resulted in mortality. This antibody also prevented clearance of VV-HA-IL2 in normal mice. However, the induction of NK cell responses in nude and normal mice and Tc cell responses in normal mice were not impaired by the Mab treatment, suggesting that the effector functions of both classes of cytolytic cells were inhibited *in vivo*.

Several lines of evidence indicated that virus-encoded IL-2 induced a possible cascade of immunological events which contributed to the rapid clearance of VV-HA-IL2 and recovery of T-cell deficient nude mice from infection.

ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cell
as-GM_1	asialo-GM_1
ß2-m	Beta 2-microglobulin
C	Complement
CMI	Cell-mediated immunity
CNS	Central nervous system
DTH	Delayed type hypersensitivity
EMEM	Eagle's minimum essential medium
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	relative centrifugal force
h	hour (s)
HBSS	Hank's balanced salt solution
HSV	Herpes simplex virus
i.p.	intra-peritoneal
i.v.	intra-venous
IFN	Interferon
IL	Interleukin
IL-2	Interleukin 2
IL-2R	Interleukin 2 receptor
LAK	Lymphokine activated killer
LCMV	Lymphocytic choriomeningitis virus
µ	microns
Mab	Monoclonal antibody
MCMV	Murine cytomegalovirus
µg	microgramme
mg	milligramme
MHC	Major histocompatibility complex
MHV	Murine hepatitis virus
min	minutes
µl	microlitres
mM	millimolar
µm	micrometres
MSV	Moloney sarcoma virus
MTD	mean time to death
NK	Natural killer

PBS	Phophate buffered saline
PE	Phycoerythrin
PFU	plaque forming units
RSV	Respiratory syncytial virus
s.c.	sub-cutaneous
SFV	Semliki Forest virus
T_c	Cytotoxic T
TCR	T-cell receptor
Td	T cell mediated delayed type hypersensitivity
T_h	Helper T
TK	Thymidine kinase
TNF	Tumour necrosis factor
VV	Vaccinia virus

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CHAPTER 1

Introduction and Literature Review

1. The immune response to viral infection: An overview

The mammalian immune system comprises a highly complex and efficient immune surveillance network which is believed to have evolved in essence to distinguish self from non-self. Host defense against viral infection occurs in three distinct but sometimes interacting phases; innate resistance that is non-inducible and immediate; an early, inducible phase that is largely antigen non-specific; and a late (relative to the first two phases) phase that is inducible, highly antigen-specific and generates immunological memory (Janeway, 1988, 1989). Natural killer (NK) cells and monocytes/macrophages are active participants in the innate and early, interferon-inducible phases of the immune response to viral infection (reviewed in Welsh, 1986; Trinchieri *et al.*, 1978; Gidlund *et al.*, 1978; Mims and White, 1984a; Biron *et al.*, 1989). The alternative complement pathway is yet another component of the non-adaptive antiviral immune effector phase. These early components exert their antiviral function before the generation of antigen-specific immune responses, namely antibody and cytotoxic T (Tc) cells.

Activation of clonally-specific T and B cells is the critical event in the generation of cellular and humoral responses which involves numerous and complex events not yet fully elucidated. Cell-mediated immunity (CMI), the function of Tc cells, is generally believed to be important in the process of recovery from most primary viral infections (Blanden, 1974; Mims and White, 1984a; Mims, 1987a) but antibody may be a contributory factor. Neutralisation of virus particles by antibody and lysis of infected cells by the classical complement pathway and antibody-dependent cellular cytotoxicity (ADCC) can become operative once virus-specific antibody is produced. Recovery from a primary viral infection is a potentially complex process involving multiple factors. Each component of the immune system may contribute to the process of recovery but the relative importance of any one of them would depend on the type of virus-host combination.

2. Non-adaptive (innate) immunity

The innate and early inducible phases of the immune system comprise NK cells, interferons (IFNs), monocytes/macrophages, the alternative complement pathway and some secretory products of macrophages (monokines) and T-cells (lymphokines) generally termed cytokines. In some instances, the antiviral functions of some of these components are influenced by others. The role of other factors such as age of the host, body temperature, nutrition, hormonal effects and pH which influence resistance to infection will not be discussed.

2.1. Natural killer (NK) cells

NK cells are large granular lymphocytes which mediate the lysis of certain tumour and virus-infected target cells in a non-MHC-restricted fashion (Kiessling *et al.*, 1975; Herberman *et al.*, 1975; reviewed in Welsh, 1986; Ritz *et al.*, 1988). NK cells, unlike T cells, do not express cell-surface CD3 or the T cell antigen receptor (TCR) heterodimers (α and β) (Lanier *et al.*, 1983; Ritz *et al.*, 1985; Lanier *et al.*, 1986; Biron *et al.*, 1987; Biassoni *et al.*, 1988). Human NK cells express a low-affinity receptor for the Fc fragment of immunoglobulin G (IgG) $Fc\gamma R$ or CD16 antigen and the NKH-1 (Leu-19) cell surface marker (Perussia *et al.*, 1984; Fitzgerald-Bocarsly *et al.*, 1988). Although murine NK cells share some cell surface antigens with T and B lymphocytes and macrophages (Burton *et al.*, 1988), they are believed to have arisen from a non-T, non-B and non-myelomonocytic lineage (Kumar *et al.*, 1989). Murine NK cells are asialo-GM_1^+ (as-GM_1^+), Thy-$1^{\pm}$, $CD3^-$, $CD4^-$, $CD8^-$ and $J11d^-$ (Hackett *et al.*, 1986). They also express one or more of the three known NK cell-specific alloantigens, NK-1, NK-2 and NK-3 (Hackett *et al.*, 1986; Burton *et al.*, 1988; Burton *et al.*, 1989).

First recognized on the basis of their cytolytic activity, NK cells are known to exert various other functions such as the production of cytokines, regulatory functions on the adaptive immune system and on haematopoiesis and natural resistance against tumours and microbial infection (Trinchieri and Perussia, 1984). Only recently has there been mounting evidence that NK cells mediate antiviral function *in vivo* during certain viral infections (reviewed in Welsh, 1986).

During systemic virus infections, NK cell responses are markedly augmented in most virus-host systems (Welsh, 1981; reviewed in Welsh, 1986). The augmentation appears to be a function of activation (MacFarlan *et al.*, 1977; Welsh and Zinkernagel, 1977; Wong *et al.*, 1977; Gidlund *et al.*, 1978) and proliferation (Biron *et al.*, 1984), both of which are induced by IFN (IFN-α and -β). Peak splenic NK cell activity and blastogenesis occur shortly after the peak in splenic IFN levels (Biron and Welsh, 1982; Welsh, 1984). The demonstration by Gidlund *et al* (1978) and Grundy *et al* (1982) that antibody to IFN-α and -β reduced significantly virus-induced augmentation of NK cell activity has provided evidence that NK cell activation was mediated by virus-induced IFN. Nevertheless, viral glycoproteins may also augment NK-cell-mediated lysis (Harfast *et al.*, 1980; Alsheikhly *et al.*, 1983; Arora and Houde, 1988) in the apparent absence of detectable IFN synthesis (Casali *et al.*, 1981). NK cells only exert potent antiviral function *in vivo* early during infection, coinciding with the kinetics of their activation, and help limit the severity and duration of acute viral infection (Bukowski *et al.*, 1984).

The most convincing evidence for the antiviral function of NK cells comes from studies using appropriate reagents to selectively deplete NK cells in mice. Administration of antibody to as-GM_1, or NK-1.1 antigens enhanced viral replication and pathogenesis in mice during influenza virus (Stein-Streilein and Guffee, 1986), vaccinia virus (VV), murine hepatitis virus (MHV), murine cytomegalovirus (MCMV), encephalomyocarditis virus (ECMV) (Bukowski *et al.*, 1983) coxsackie-B3 virus (Godeny and Gaunt, 1986) and ectromelia virus (Jacoby *et al.*, 1989) infections. Further evidence for NK cell-mediated antiviral activity come from adoptive transfer studies. Bukowski *et al* (1985) found that adoptive transfer of normal spleen cells, depleted of T cells and enriched for NK cells, or cloned NK cells conferred protection in newborn mice against MCMV infection. The protective effects of the transferred cells were abolished when mice were treated with antibody to as-GM_1. Using similar procedures, NK-1.1$^+$, as-GM_1^+ NK cells were shown to confer protection against herpes simplex virus-1 (HSV-1) infection in mice (Rager-Zisman *et al.*, 1987). A recent report (Biron *et al.*, 1989) that severe varicella virus infection followed by CMV infection developed in an individual with intact cell-mediated and humoral immune functions but

otherwise completely lacked $CD16^+$, $NKH\text{-}1^+$ NK cells suggests that these effectors may be important in the control of some human viral infections.

Apart from enhanced splenic NK activity, an increase in NK activity and cell number is demonstrable in organs which harbour viral replication (Welsh, 1978; Doherty and Korngold, 1983; Leung and Ada, 1982; Stein-Streilein *et al.*, 1983). The accumulation of NK cells at sites of viral replication has been attributed to their ability to respond chemotactically to IFN-β and IL-2, two factors that are produced by infected cells and T cells respectively (Natuk and Welsh, 1987). Presumably, the accumulation of activated NK cells at the sites of virus infection is essential for mediating their antiviral function.

An paradoxical feature of the action of IFNs is that they enhance the cytotoxic activity of NK cells while antagonistically protecting target cells from NK cell-mediated lysis (Welsh, 1978; Gidlund *et al.*, 1978; Trinchieri and Santoli, 1978). The induction of resistance in targets against lysis is an active process requiring RNA and protein synthesis and therefore cells infected with cytopathic viruses that turn-off host RNA and protein synthesis are not protected from NK cell mediated lysis. Such a mechanism would ensure that only virally infected cells are eliminated by NK cells. The insensitivity to NK cell-mediated lysis has been attributed to membrane alterations induced by IFN treatment of targets which include marked changes in cell surface glycoproteins (Lindahl *et al.*, 1976; Minato *et al.*, 1980), glycosphingolipids and sialic acid (Yogeeswaran *et al.*, 1981; 1982; 1983). Increase in cell surface sialic acid expression on target cells has been inversely correlated with sensitivity to NK cell-mediated lysis (Yogeeswaran *et al.*, 1982). In particular, IFN-induced upregulation of MHC antigens is generally believed to influence NK recognition of targets. In a number of studies (Pointek *et al.*, 1985; Harel-Bellan *et al.*, 1986; Storkus *et al.*, 1987; Storkus *et al.*, 1989), an inverse relationship between the concentration of class I MHC antigen expression on target cell surface and target cell susceptibility to NK cell-mediated lysis has been observed. This led to the hypothesis that class I MHC antigens deliver a negative signal to NK cells (the converse is true for Tc cells). The validity of this hypothesis has been challenged by a number of other studies (Chervenak and Wolcott, 1988; Dennert *et al.*, 1988; Gorelik *et al.*, 1988; Mullbacher and King, 1989) which have failed to demonstrate any correlation between increased class I MHC antigen expression and a

concurrent decrease in NK cell-mediated lysis. Two other reports (Reiter *et al.*, 1988; quoted in Lotzova and Ades, 1989) also support the contention that susceptibility of targets to NK cell-mediated lysis is not dependent on the concentration of class I MHC antigen *per se* . Firstly, expression of HLA-A2 antigen following gene transfection into the HLA-negative K562 (NK sensitive) cells did not affect NK cell-mediated lysis (quoted in Lotzova and Ades, 1989). Secondly, the demonstration that IFN-treated targets were protected from lysis by NK cells without the induction of any class I MHC antigen expression (Reiter *et al.*, 1988) clearly suggests that other factors may also influence susceptibility.

NK cells do not require prior sensitization or antigen presentation to be activated. They are not restricted by class I nor class II MHC antigens and it is conceivable that they may recognize a number of different ligands or some relatively common cell surface change, in view of their ability to lyse a wide range of targets. One such ligand appears to be β2-m (Mullbacher and King, 1989), a molecule which is non-covalently attached to class I MHC antigen molecules expressed on the cell surface and in most cases is required for the surface expression of the class I antigens. However, class I MHC-unassociated (free) β2-m is known to be present on the cell surface (Solheim and Thorsby, 1974), presumably via inositol linkage. Mullbacher and King (1989) used the HLA-negative K562 cells which otherwise express β2-m to address the role of this ligand in NK recognition. IFN-γ treatment of these cells increased β2-m expression and there was a concurrent increase in susceptibility to NK cell-mediated lysis. It is relevant that another cell line, Daudi, which expresses neither β2-m or class I antigen was not susceptible to NK cell-mediated lysis even after treatment with IFN-γ. Mab to human β2-m was shown to inhibit NK cell-mediated lysis of K562 targets. In addition, mouse embryo fibroblasts, which express little or no class I MHC and β2-m molecules, infected with a recombinant VV encoding human β2-m had moderately increased concentrations of cell surface β2-m and concurrently exhibited increased susceptibility to NK cell-mediated lysis. These authors concluded that class I MHC-unassociated β2-m serves as a ligand for NK cell-mediated lysis. While class I MHC and β2-m expression must be co-regulated to some extent, it is possible that there could be variations in the ratios of the two different molecules in different cell classes, thus allowing the possibility that free β2-m would be available as a ligand for NK cell recognition. It is well documented that adenoviruses specifically

interfere with the expression of cell surface class I MHC antigens (Eager *et al.*, 1985; Burgert and Kvist, 1985; Andersson *et al.*, 1985; Paabo *et al.*, 1986), but not β2-m (Eager *et al.*, 1985). The reduced expression of class I MHC antigens on adenovirus-infected cells has indeed been shown to reduce the efficiency of Tc cell recognition (Anderson *et al.*, 1987). Whether or not NK cell-mediated lysis of adenovirus-infected targets is enhanced because of the availability of class I MHC-unassociated β2-m is not known but the hypothesis that β2-m may be a ligand for NK cells can be readily tested using adenovirus-infected cells.

Infection with lymphocytic choriomeningitis virus (LCMV) efficiently induces IFN production and NK cell activation in mice. However, all the available evidence indicates that NK cells do not play any role in providing protection against this virus (Welsh and Kiessling, 1980; Welsh *et al.*, 1984; Bukowski *et al.*, 1983). LCMV does not shut-off host RNA and protein synthesis and hence IFN-treated, LCMV-infected cells, like uninfected cells, are rendered resistant to NK cell-mediated lysis (Trinchieri and Santoli, 1978; Trinchieri *et al.*, 1981; Bukowski and Welsh, 1985; Welsh *et al.*, 1981; de Fries and Golub, 1988). While IFN-induced target cell protection would ensure that uninfected cells are not lysed indiscriminately, it would also prevent elimination of non-cytopathic viruses.

2.2. Mononuclear phagocytes

The mononuclear phagocyte system consists of blood monocytes and tissue macrophages which are strategically placed in the various body compartments. They are not merely scavenger cells, phagocytosing and degrading infectious material but possess both intrinsic and extrinsic antiviral properties which may determine the outcome of an infection (Mims and White, 1984b). Their essential role as antigen presenting cells (APCs) in the induction of antigen-specific immune responses will be discussed in section 3.2.4. Macrophages have virucidal, cytotoxic, cytostatic and cytokine secreting potentials and these depend on the genetics and age of the host and the state of differentiation and activation of the cells (Morahan, 1980). Additionally, macrophages from different sites in the body may display quite distinct antiviral properties (Rodgers and Mims, 1981), as may subpopulations from any given site which may differ in susceptibility to infection (Stueckemann *et al.*, 1982).

It has been clearly established that susceptibility to infection with a number of viruses is dependent on the outcome of virus-macrophage interactions (Mims, 1964). For instance, the inability of blood monocytes to take up polio virus type I favours persistence of viraemia. Non-pathogenic viruses like the CL strain of VV or sublethal doses of influenza virus are destroyed by macrophages when injected intravenously into mice. Infection of mice with myxoma virus (a natural rabbit pathogen) and rats with ectromelia virus (a natural mouse pathogen) also result in abortive infection of macrophages and infection does not proceed. In some instances, uptake of virus and passive transfer to hepatic cells can occur as in the case of Rift Valley fever virus (Mims, 1957). When liver macrophages are productively infected, release of progeny leads to the infection of hepatic cells as can be seen in mousepox (Mims, 1959) and probably in man with variola and yellow fever viruses. The studies by Roberts (1963) with avirulent and virulent strains of ectromelia virus produced strong evidence that virulence was associated with the ability of virus to productively infect macrophages. After intravenous injection, the avirulent strain of virus infected liver macrophages with much greater difficulty and macrophages yielded significantly fewer viral particles than in the case of the virulent strain. The ability of ectromelia virus to infect hepatic parenchyma cells is a prerequisite for the pathogenesis of mousepox and is dependent on the susceptibility to infection of the liver macrophages; these in turn transmit infection to adjacent hepatocytes (Mims, 1959). Once growth in hepatic parenchymal cells was established, no differences between the avirulent and virulent mousepox virus strains could be detected (Roberts, 1963). Thus, in an infectious process where the outcome depends on the race between virus growth in liver parenchyma on the one hand, and the immune response on the other, a delayed initiation of viral replication in a major target organ is crucial for the recovery process.

Monocyte/macrophage depletion in mice may be achieved by treatment with silica . This approach has been used quite extensively to evaluate the antiviral role of mononuclear phagocytes *in vivo* (Allison, 1976). Adult mice survive i.v. or i.p. injections with HSV-1 but elimination of macrophages with silica resulted in lethal hepatitis (Zisman *et al.*, 1970). Furthermore, clearance of yellow fever virus from the circulation of silica-treated mice was significantly delayed (Zisman *et al.*, 1971). A

large proportion of virus inoculated i.p. is inactivated by peritoneal macrophages and hence a substantially higher dose of virus is needed to establish infection if inoculated through the i.p. than i.v. route (Mims, 1964). However, following i.p. treatment with silica, much lower doses of Friend virus, SFV and ECMV were required to establish infections (Zisman *et al.*, 1970; Larson *et al.*, 1972).

Studies by Bang and Warwick (1960) suggested that the genetic difference in susceptibility of two strains of mice to MHV infection operated at the macrophage level. Haller (1981) showed that macrophages from various strains of mice exhibit differences in susceptibility to influenza virus infection. Resistance is dependent on the presence of the *Mx* gene, the expression of which is controlled by IFN-α.

Other antiviral functions of macrophages include ADCC (MacFarlan and White, 1983; Smith and Sheppard, 1982) and the participation in delayed type hypersensitivity reaction mediated by T cells (see section 3.2.7.).

Most macrophages produce IL-1 (Oppenheim *et al.*, 1979) and there has been one report (van Damme *et al.*, 1987; Billau, 1987) on the antiviral activity of IL-1 which is thought to be mediated through the secretion of IFN-β. IL-1 also participates in the inflammatory response as an inducer of inflammatory mediators (Mizel *et al.*, 1981), acute phase proteins (Stzein *et al.*, 1981) and fever (Murphy *et al.*, 1980) which could all contribute to antiviral mechanisms.

2.3. Antiviral factors

2.3.1 The interferons

The production of interferons (IFNs) is a regular feature of virus infections. Apart from their role in NK cell activation and proliferation, the IFNs are perhaps the best characterized family of proteins with known antiviral activity both *in vivo* and *in vitro.*

There are three antigenically distinct species of IFNs, namely alpha IFN (IFN-α), beta IFN (IFN-β) and gamma IFN (IFN-γ). The type of IFN that is induced is dependent on the infecting virus and the interacting cell involved (Stanton and Baron, 1984). Both IFN-α and -β are produced by leukocytes (T,B, null and macrophages), epithelial cells and fibroblasts following viral infection whereas IFN-γ is produced mainly by activated

T cells after antigen-specific stimulation (Interferon nomenclature, 1980). Mitogens can also induce T cells to secrete IFN-γ and NK cells are known to produce IFN-γ (Handa *et al.*, 1983; Sandvig *et al.*, 1987; Young and Ortaldo, 1987). Under appropriate experimental conditions, mice with severe combined immunodeficiency (SCID) with no functional T- and B-cells and athymic, nude mice can produce this cytokine (Bancroft *et al.*, 1987; Wentworth and Ziegler, 1987).

IFNs are the earliest of the known mammalian inducible host-defense mechanisms and become operative within hours of infection. The induction of an antiviral state in uninfected cells is related to the capacity of IFNs to induce the production of a number of enzymes which may play important roles in the regulation of viral and cellular macromolecular synthesis and degradation (reviewed in Revel and Chebath, 1986; reviewed in Pestka and Langer, 1987). The ability of one or more of these enzymes to render any cell resistant to infection is dependent on the type of cell, the virus and the type of IFN. These parameters would determine the effectiveness of such a mechanism to limit viral spread.

Earlier studies by Gresser and others (1976) suggested that IFN-α and -β contributed to antiviral function *in vivo*. They demonstrated that administration of antibodies to IFN-α and/or -β increased the severity of infections with HSV, Moloney sarcoma virus (MSV), vesicular stomatitis virus (VSV), Newcastle disease virus (NCDV) and ECMV in mice but infection with influenza virus was unaffected. In the light of these findings, it was thought that influenza virus was insensitive to the antiviral action of IFNs. However, there is a distinct possibility that the anti-IFN antibody administered i.p. or i.v. may not have reached the tracheobronchial epithelium (site of influenza virus replication) in adequate concentrations to affect local IFN activity. A point of relevance in this regard is the finding that influenza virus inoculated i.n. in mice was not affected by NK-cell depleting antibody given i.v. but infection was significantly exacerbated if antibody was administered intranasally (Stein-Streilein and Guffee, 1986). It therefore seems that viruses differing in the preferred site of replication (different organs) may require different antibody treatment procedures to demonstrate the antiviral activity of IFN or other components of the immune system.

A recent report by Leist *et al.* (1989) highlights the possible antiviral role of IFN-γ *in vivo*. Administration of anti-IFN-γ antibody enhanced titres of VSV and VV in normal mice. Similarly, titres of LCMV were significantly increased in nude and normal mice treated with IFN-γ antibody. Antiviral Tc cell responses to VV and VSV were not affected by antibody treatment but anti-LCMV Tc cell response was severely impaired. The impaired Tc cell response to LCMV was thought to be partly due to 'high-dose immune paralysis' phenomenon previously postulated by Hotchin (1971). Similar studies by Wille *et al.* (1989) led them to suggest that the impaired anti-LCMV Tc cell response was not due to enhanced LCMV titres in anti-IFN-γ antibody treated mice, but rather, the anti-IFN-γ antibody inhibited the generation of Tc cell response. The general findings of both groups are similar, i.e. treatment with anti-IFN-γ antibody resulted in increased LCMV titres and impaired the Tc cell response, but they differ in the interpretation of their respective results. The crucial point is increased LCMV titres and impaired Tc cell response always go together. The question then is "what is cause and what is effect?" The two possible explanations are: i) titres of LCMV increase because Tc cell response is impaired by treatment with anti-IFN-γ; ii) Tc cell response is impaired because anti-IFN-γ antibody inhibited clearance of LCMV which consequently affected Tc cells. If the former explanation were true, then Tc cell responses to VV and VSV should also have been impaired, but they were not. The latter explanation seems more likely because LCMV titres in T cell-deficient nude mice increased significantly following anti-IFN-γ antibody treatment (Leist *et al.*, 1989). Adoptive transfer of LCMV-immune Tc cells into virus-infected and IFN-γ antibody-treated mice should provide the evidence consistent with the above argument.

To address the role of IFN-γ in antiviral immunity, Kohonen-Corish *et al.* (1990) inserted the murine IFN-γ (Mu-IFN-γ) gene into VV genome. The recombinant virus, designated VV-Mu-IFN-γ, was found to be highly attenuated in athymic nude mice and euthymic normal mice. Nude mice survived an infection with VV-Mu-IFN-γ but not with a control recombinant VV not encoding Mu-IFN-γ or another recombinant VV encoding human IFN-γ (IFNs are strictly species specific). VV-Mu-IFN-γ was cleared from ovaries of normal mice by day 4 post-infection whereas between 6-8 $\log_{10}$ PFU of the control viruses were recovered from ovaries

at day 5. The mechanism of rapid VV-Mu-IFN-γ clearance was not mediated by antibody, NK cells or anti-VV Tc cells. The available evidence suggested that virus-encoded murine IFN-γ most likely contributed to viral clearance through direct antiviral activity. Based on these observations, the authors raised the possibility that the major function of immune Tc cells (known to produce IFN-γ) was to focus the secretion of antiviral cytokines like IFN-γ at sites of viral infections. While this speculation has some basis, it is clear that IFN-γ has other known functions which could potentially contribute to the process of virus clearance. Apart from its capacity to induce an antiviral state in uninfected cells, IFN-γ can activate mononuclear phagocytes to increase their virucidal capacity as well as upregulate the expression of class I MHC antigen expression (see below) on targets which could increase the sensitivity of Tc cell recognition. The kinetics of VV-Mu-IFN-γ clearance in that study suggests the possibility that virus-encoded IFN-γ, produced early in infection, may have activated mononuclear phagocytes which contributed to virus clearance. Data presented in Chapter 5 is consistent with this argument. The role of IFN-γ in antiviral immunity will be discussed further in section 3.2.5.1. and Chapter 5.

Apart from their direct antiviral function, IFNs also play an important role in immunoregulation which could determine the outcome of a viral infection or disease. All the three species of IFNs upregulate the expression of class I MHC antigens and β2-m , but IFN-γ is the most potent and is the only IFN that upregulates the expression of class II MHC antigens. IFN-induced expression of class 1 MHC antigens and β2-m on virus-infected cells may contribute to antiviral mechanisms possibly by enhancement of Tc (Bukowski and Welsh, 1985; King *et al.*, 1986) and possibly NK (Mullbacher and King, 1989) cell recognition. Additionally, IFN-γ-induced expression of class II MHC antigens on some cell types such as macrophages and Langerhans cells has possible immunological implications particularly with regard to antigen presentation (reviewed in Janeway *et al.*, 1984). Thus, if the basal level of the cell-surface histocompatibility antigens is below the threshold concentration normally required for efficient T cell responses, then the IFN-induced increase in levels of class I and class II antigens could represent an important component of the host response to virus infections. It should be emphasized that the threshold level may vary in different experimental systems.

2.3.2. Tumour necrosis factors-α and -β

The production of TNF-α, first described as a factor that caused necrosis of tumours (Carswell *et al.*, 1975), is triggered by a variety of stimuli including viruses (Aderka *et al.*, 1986; Wong and Goeddel, 1986). Although macrophages are the principal TNF-α producers (Carswell *et al.*, 1975), mast cells (Young *et al.*, 1987) and activated T cells following mitogenic stimulation (Turner *et al.*, 1987) may also produce this cytokine.

Recently, TNF-α was shown to have intrinsic antiviral activity on some cell types *in vitro* (Kohase *et al.*, 1986; Mestan *et al.*, 1986; Wong and Goeddel, 1986), and to act synergistically with the antiviral activity of IFN-α, -β and -γ on a variety of cell types against a number of RNA and DNA virus infections (Wong and Goeddel, 1986; Mestan *et al.*, 1988; Reis *et al.*, 1989). Furthermore, TNF-β can selectively lyse virus-infected but not uninfected cells *in vitro* (Aderka *et al.*, 1985). This mode of action is believed to be related to the known increased cytocidal activity of the cytokine in cells treated with inhibitors of cellular RNA or protein synthesis. This suggests that only viruses that shut-off host RNA or protein synthesis would make infected targets susceptible to lysis. Wong and Goeddel (1986) have also shown that TNF-α inhibits replication of some RNA and DNA viruses in the absence of an apparent contribution of endogenous IFNs. They further demonstrated that TNF-β (lymphotoxin) also possessed antiviral activity *in vitro* which could be greatly enhanced in synergy with TNF-α. It is interesting to note that each of the soluble mediators described so far have intrinsic antiviral properties which can be amplified by the synergistic interactions with another of these factors. The precise *in vivo* antiviral role of TNF-α and -b have not been elucidated.

2.4. The alternative complement pathway

The alternative or properdin pathway of the complement system is essentially a bypass system for the classical pathway and may not require the presence of antibody (reviewed in Hirsch, 1982).

Several viruses or virus-infected cells, including Sindbis, VSV, measles and RSV (Smith *et al.*, 1981; reviewed in Hirsch, 1982) directly activate the alternative complement pathway. Because this pathway is antibody-

independent, it can be activated immediately after viral invasion, before antigen-specific immune responses have been generated. Evidence for a precise role for complement in antiviral function in humans is lacking.

3. Adaptive Immunity

3.1. The major histocompatibility complex (MHC)

The murine major histocompatibility gene complex (*H-2*) comprises the *K*, *I* and *D* regions (Klein, 1975; Snell *et al.*, 1976; reviewed in Klein, 1986). The *K* and *D* regions encode the class I (K and D) glycoproteins whereas the *I* region encodes the class II (Ia) glycoproteins . The class I antigens are expressed virtually on all somatic cells but class II antigen expression is limited to certain cell types like B cells (Sachs and Cone, 1973), dendritic cells (Steinman, 1981), some populations of macrophages (Beller and Unanue, 1981) Langerhans cells (Freilinger *et al.*, 1981) and intestinal epithelial cells (Parr and McKenzie, 1979). The MHC gene products restrict the activities of T cells with specificity to foreign antigens.

3.2. T Lymphocytes

Thymus derived (T) lymphocytes may be distinguished from bone marrow or bursa derived (B) lymphocytes by the presence of antigens recognizable by monoclonal antibodies (Mab). In man these are the OKT, CD4 and CD8 antigens and in mice the Thy-1, Ly and Qa antigens (reviewed in McKenzie and Potter, 1979; Sutton *et al.*, 1989). Although the Thy-1 antigen is expressed by all T cells, it is not exclusively a T-cell antigen since some nonhaematopoietic cells (Reif and Allan, 1964; reviewed in Mckenzie and Potter, 1979) and NK cells (Hackett et al., 1986) are Thy-1^+.

T cells have been classified into subsets according to surface antigen expression and function. The two main subsets of peripheral T cells in the mouse are $Ly1^+2^-3^-$ (Ly1) and $Ly1^-2^+3^+$ (Ly2,3). Using antisera directed against Ly1 and Ly2, T cells mediating DTH and involved in T-B cell collaboration were found to be of the $Ly1^+2^-$ phenotype whereas most $Ly1^-2^+$ T cells had a cytotoxic function (Kisielow *et al.*, 1975; Cantor and Boyse, 1975). With the advent of more sensitive methods like flow cytometry, Ly2,3 T cells were found to express low levels of the Ly1

molecules (Mathieson *et al.*, 1979; Ledbetter *et al.*, 1980) and therefore Ly1 is not a suitable marker for differentiating T lymphocyte subsets .

Subsequently, a Mab which recognized an antigen designated L3T4 was found to be expressed solely on $Ly1^{+}2^{-}$ T cells (Dialynas *et al.*, 1983a, b). Using the CD nomenclature, the antigenic determinant defined by L3T4 has been designated CD4 while the Ly2 antigen has been designated CD8. The $CD4^{+}$ T cell population includes subsets that mediate helper/inducer functions (Th), DTH (Td) and in some instances cytotoxicity. Recently, Th cells have been further subdivided into Th1 and Th2 on the basis of different patterns of cytokine secretion and function (Mosmann *et al.*, 1986; reviewed in Mosmann and Coffman, 1989). The Th1 subset mediates DTH and corresponds functionally to Td. The Th2 subset is involved in helper functions. $CD8^{+}$ T cells mediate cytotoxic (Tc) functions. Suppressor T (Ts) cells may be $CD4^{+}$ or $CD8^{+}$ and regulate the antigen-specific immune response by inhibiting the effector functions of Th, Td, and Tc (Gershon and Kondo, 1970; Germain and Benacerraf, 1981; reviewed in Klein, 1986) and will not be discussed here.

The majority of peripheral $CD4^{+}$ and $CD8^{+}$ T cells in the mouse express the αβ-TCR but a small proportion of T cells express the γδ-TCR and are mainly $CD4^{-}$, $CD8^{-}$ (Brenner *et al.*, 1986; Bank *et al.*, 1987; reviewed in Brenner *et al.*, 1988; Janeway *et al.*, 1988; Bluestone and Matis 1989). It is not known if $\gamma\delta\text{-TCR}^{+}$ T cells have any antiviral function. The TCR heterodimers (αβ or γδ) are usually associated with other molecules of the CD3 complex (reviewed in Clevers *et al.*, 1988).

3.2.1. Antigen recognition and MHC-restriction of T cells

The induction of an antigen-specific immune response is dependent on antigen recognition by clonally-specific T and B cells. T and B cells "perceive" antigens in completely different ways.

Antigen-specific B cells recognize and bind to epitopes dependent upon protein or carbohydrate structure via surface immunoglobulin (sIg) (Wigzell and Makela, 1970; Greaves and Hogg, 1971; reviewed in Benjamin *et al.*, 1984). Two classes of protein B-cell epitopes are known. Conformational epitopes are defined as those which are dependent on the native spatial conformation of protein whereas sequential epitopes are those dependent only on the amino acid sequence of the corresponding

peptide segment (Sela *et al.*, 1967; Sela, 1969). However, B-cell binding to either class of epitopes is not different. These epitopes are regarded as topographic, i.e. they are composed of structures on the protein surface. The antibody synthesized following B cell activation binds to protein antigen based on its original conformation as originally "perceived" by sIg on the B cell.

T cells, on the other hand, recognize antigen only when it is presented on the surface of other cells in association with appropriate class I or class II MHC molecules, a phenomenon known as MHC restriction. Antigen recognition requires intracellular degradation of protein (antigen processing), association with MHC molecules and presentation to T cells by antigen presenting cells. Recent evidence suggests that foreign antigens recognized by T cells are processed intracellularly into short peptides (Shimonkevitz *et al.*, 1983) prior to association with MHC molecules. Hence, peptides derived from processed antigen in APCs or target cells (Babbit *et al.*, 1985; Townsend *et al.*, 1986a) bind to the MHC molecules and the peptide-MHC molecule complex is presented on the surface of these cells.

3.2.2. Antigen processing

At least two distinct pathways of antigen processing exist (reviewed in Morrison *et al.*, 1986a, b; Braciale *et al.*, 1987a; Long, 1988; Long, 1989). These pathways, depending on the nature of antigens, i.e., endogenous or exogenous, determine whether antigen is recognized by class I- or class II-restricted T cells. Class I MHC antigens are restriction elements for $CD8^+$ T cells and class II MHC antigens are restriction elements for $CD4^+$ T cells.

The primary requirement for entry of antigen into the class I pathway of processing and presentation is a cytoplasmic localization. In addition to cellular proteins undergoing normal degradation, this requirement may also be met during viral infection. Class I-restricted T cells recognize peptides derived from endogenous viral protein synthesis (Townsend *et al.*, 1986a, b; Braciale *et al.*, 1987a, b). Such peptides complex with *de novo* synthesised and/or recycling class I MHC molecules before presentation on the surface of APCs or target cells. Most somatic cells express class I molecules and all class I-bearing cells are thought to be capable of antigen presentation although some lack "signal 2" for primary T cell

activation (Andrus *et al.*, 1980; Lafferty *et al.*, 1980; Lafferty *et al.*, 1983; Lafferty, personal communication) (see section 3.2.4.)

For exogenous antigens presented in the context of class II MHC molecules, intact antigens enter the cell by means of endocytic vesicles either by pinocytosis or receptor-mediated endocytosis . Endocytosed antigen is degraded in acidic lysosomes. The resulting peptides are complexed with class II MHC molecules and then expressed on the surface of Ia^+ APCs (reviewed in Schwartz, 1985; Germain, 1986). Thus, the antigens processed and presented by Ia^+ APCs, e.g. B cells, dendritic cells and macrophages, are only seen by class II-restricted T cells. There may be exceptions to this rule as demonstrated by the observation that some exogenous antigens can be presented by class I molecules (Staerz, 1987; Yewdell *et al.*, 1988). Fusion of non-infectious influenza virus with cell membrane causes viral proteins to enter the class I pathway of antigen processing (Yewdell *et al.*, 1988)

The generally accepted concept that $CD4^+$ and $CD8^+$ T cells recognize processed forms of protein antigens together with MHC molecules is consistent with evidence that both types of cells share a common pool of TCR V genes (reviewed in Kronenberg *et al.*, 1986; Davis and Bjorkman, 1988) which belong to the immunoglobulin supergene family. The reasons for $CD4^+$ T cells being class II MHC-restricted and $CD8^+$ T being class I MHC-restricted therefore probably resides in the fact that CD4 binds to class II MHC and CD8 binds to class I MHC and that this binding is usually required for T cell activation (Doyle and Strominger, 1987; Ratnofsky *et al.*, 1987).

Because T cells have only one receptor (TCR) that accounts for both antigen and MHC specificity (Dembic *et al.*, 1986), the association of peptide (usually of 6-20 amino acid residues) and the MHC molecule becomes a crucial factor in the initiation of the immune response. It should be emphasised here that the specificity of T cell recognition is governed by the peptide-MHC molecule complex (epitope) and not the peptide alone.

3.2.3. MHC restriction of Th and Tc cells

In the induction of an antibody response, antigen-specific B cells interact with antigen-specific Th cells (Mitchison, 1971). This interaction is MHC-restricted (Kindred and Shreffler, 1972; Katz *et al.*, 1973). Antigen bound by the B cell is internalized and processed as by other APCs and displayed on the surface in association with class II MHC molecules. The antigen-specific, class II-restricted $CD4^+$ Th cells that recognize peptide plus MHC are triggered to produce B cell growth factors which result in the clonal expansion of antibody producing B cells. The importance of MHC restriction was also demonstrated in macrophage-Th cell interactions (Rosenthal and Shevach., 1973).

The subsequent demonstration by Zinkernagel and Doherty (1974) that virus-immune Tc cells killed infected targets only if the effector and target cell types shared the same class I MHC (K/D) antigens not only indicated that Tc cells were MHC-restricted but also the provided a basis for understanding the role and *in vivo* significance of MHC encoded antigens and the function of Tc cells in antiviral immunity (reviewed in Zinkernagel and Doherty, 1979).

3.2.4. T-cell activation

The development of the adaptive-immune response to viral infection requires the activation of B, Th and Tc cells. This results in the clonal expansion of these antigen-specific lymphocytes and their differentiation to acquire effector function manifested either by secretion of bioactive molecules (e.g. cytokines or antibody) or the development of cytotoxic function. This review will be confined to activation of T cells only.

The most crucial event in T cell activation is the interaction of the TCR/CD3 complex with the antigen-MHC molecule complex. If this binding achieves the required threshold load, protein kinase C activation is triggered in the T cell and production of inositol 1,4,5-triphosphate then mobilizes intracellular calcium. This is followed by the secretion of T cell-derived factors (cytokines) involved in proliferation, differentiation and activation of lymphocyte subsets (see below). There is evidence that CD4 and CD8 molecules participate as crucial accessory molecules in the interactions of T cells with the APCs (Gabert *et al.*, 1987). Their contribution is particularly important when the binding between peptide-

MHC complex and TCR is of low affinity. Other molecules like ICAM-1 and LFA-1 also participate as accessory molecules in the binding of T cells to APCs (Dustin *et al.*, 1988).

3.2.4.1. Th cell activation

The antigen-specific activation of Th cells is dependent upon two distinct but interdependent APC signals. APCs are required to process and present antigen to Th cells. This function is normally a property of an Ia^+ subpopulation of macrophages, dendritic cells and B cells. Binding of the TCR, by the peptide-Ia molecule complex constitutes the first signal. The second macrophage-derived T cell activating signal (co-stimulatory signal) is provided by interleukin-1 (IL-1) (Aarden *et al.*, 1979) that is apparently produced by all macrophages (Oppenheim *et al.*, 1979) though not all Th cells require IL-1 (Kurt-Jones *et al.*, 1987; Greenbaum *et al.*, 1988; Weaver *et al.*, 1988). The nature of other possible co-stimulators in T-cell activation by APCs such as B cells and dendritic cells is currently unknown. One of the major functions of IL-1 in T-cell activation is induction of the synthesis and secretion by T cells of the T-cell growth factor, IL-2, and expression of the IL-2 receptor (IL-2R) (Smith *et al.*, 1980a; Larsson *et al.*, 1980; Waldman, 1986; Smith, 1988; Weiss, 1989). Secretion of IL-2 and expression of IL-2R by T cells occur for a finite time interval after TCR binding to epitopes on APC and IL-1 action. The subsequent utilization of IL-2 dictates the progression of only the responsive (and hence antigen-specific) T cells from the G_0 to G_1 and eventually the S phase of the cell cycle (Stern and Smith, 1986). The ultimate determinant of the magnitude of any response, however, is a function of the concentration of T cell epitopes (peptide-Ia complexes) on APCs (Matis *et al.*, 1983) which trigger the cascade of events outlined above.

3.2.4.2. Tc cell activation.

Most of the inferences made regarding requirements for induction of Tc cell responses have been drawn from observations of alloreactive Tc cell generation *in vitro*. Also, the primary and secondary T cell activation requirements may be different (Lafferty *et al.*, 1980).

Initiation of $CD8^+$ Tc cell activation requires binding of the TCR to peptide-class I MHC molecule complexes on APCs or target cells as the first

signal. Earlier studies (Wagner *et al.* 1973; Bach *et al.* 1976; Larsson *et al.*, 1980) have suggested that at least 2 signals are necessary for the generation of alloreactive Tc cells from precursors. The co-stimulatory signal may be provided by IL-1 in the case of APCs but it is not clear if non-lymphoreticular cells can provide such a signal or if indeed IL-1 is the only such signal required. That the second signal be provided by IL-2 is widely accepted (Wagner *et al.*, 1980a; Smith *et al.*, 1980b; Lafferty *et al.*, 1980; Lafferty *et al.*, 1983). However, there have been indications that a third signal may be involved for Tc cell activation (Farrar *et al.*, 1981; Raulet and Bevan, 1982; Falk *et al.*, 1983; Kanagawa, 1983; Wagner and Hardt, 1986). Farrar *et al* (1981) proposed that induction of Tc cell response involves a linear cell-factor interaction in which IL-1 (macrophage derived) stimulates T cells to produce IL-2, which in turn stimulates T cells to produce IFN-γ. IL-2 alone can induce proliferation and differentiation but IFN-γ may be important to acquire cytolytic function. That the third signal may be IFN-γ was also suggested by a number of other reports (Simon *et al.*, 1979; Klein and Bevan, 1983; Giovarelli *et al.*, 1988; Siegel, 1988). According to Klein and Bevan (1983), IL-2 is a requisite signal not only for the development of Tc effector function but also for the production of IFN-γ, and that Tc effector function and IFN-γ release are closely associated immunologic events. Because generation of alloreactive Tc cell responses could be ablated by antibody to IFN-γ (Simon *et al.*, 1986; Siegel, 1988; Giovarelli *et al.*, 1988), these reports suggested that IFN-γ is neccessary for the induction of cytolytic activity but some others (Bucy *et al.*, 1988) do not share this view.

Tc cell activation can occur without $CD4^+$ Th-Tc cell interaction (Mizuochi *et al.*, 1985; reviewed in Singer *et al.*, 1987) and the helper function in the generation of allogeneic Tc cell responses can be provided by a $CD4^-CD8^+$, lymphokine secreting population of T cells. Anti-VV Tc cell responses can be generated *in vitro* in the absence of $CD4^+$ T cells but in the presence of IL-2 secreting $CD8^+$ T cells (Mizuochi, 1989) (see section 3.2.6). There are indications that IL-4, IL-5 and IL-6 may also be involved in Tc cell activation as demonstrated *in vitro*. IL-4 and IL-5 are known B-cell stimulatory and differentiation factors (Kishimoto, 1988). Virus infection stimulates the production of IL-6 (IFN-β2), a cytokine that is produced by fibroblasts, endothelial cells, monocytes/macrophages, T and B cells (Mizel, 1989). *In vitro*, this cytokine is known to stimulate T cell proliferation, induce IL-2R expression and induce the generation of Tc

cell responses. Whether IL-4, IL-5 and IL-6 are involved in Tc cell generation *in vivo* is yet to be elucidated.

3.2.4. Cytokines and regulation of the immune response

Apart from the cytokines described above, a variety of others including IL-3, IL-5, IL-7, TNF-α and -β, and colony stimulating factors (CSF) regulate the proliferation, differentiation, maturation and activation of lymphoid and non-lymphoid cells involved in the immune response (Mizel, 1989). Activation of cytokine gene expression is rapid and transient. The inflammatory response that requires cell proliferation and activation may be restricted to the site of cytokine production, since these soluble factors appear to be released in small amounts in a rather localized space in the vicinity of interacting cells so that effective concentrations only occur near the site of production (Poo *et al.*, 1988). In addition, the production and activity of cytokines are tightly regulated. The triggering of cytokine production in the process of T cell activation in viral infections is a function of epitope (formed by a complex of viral peptide and MHC) concentration (Sinickas *et al.*, 1985). Hence, elimination of virus (antigen) will eliminate the trigger and lead to termination of the response.

3.2.5. Cytotoxic T (Tc) cells

Cytotoxic activity of a T lymphocyte is triggered by binding of the TCRs to epitopes on the target cells (or by binding of mitogen to the T cells). Once this occurs, the Tc cells can lyse any cell displaying the relevant epitope in the near vicinity. Tc cell responses can be made to allogeneic or xenogeneic cells, to syngeneic cells bearing chemically modified determinants or to virus infected or transformed cells (reviewed in McKenzie and Potter, 1979; reviewed in Klein, 1986). Tc cells have clearly demonstrable antiviral function *in vivo*. The classical antiviral Tc cells are $CD8^+$ and recognize viral peptides on the surface of infected cells in association with self class I MHC molecules (Zinkernagel and Doherty, 1974; Blanden *et al.*, 1975; reviewed in Zinkernagel and Doherty, 1979; Swain and Dutton, 1980; Townsend *et al.*, 1986a, b). Recently, the existence of class II MHC-restricted, $CD4^+$ Tc cells have been described but their importance in antiviral immunity is unclear (Meuer *et al.*, 1983; Misko *et al.*, 1984; Morrison *et al.*, 1986a, b; Jacobson *et al.*, 1984).

3.2.5.1. Classical CD8$^+$ Tc cells

The importance of class I MHC-restricted antiviral Tc cells in recovery from primary viral infections is well recognized (reviewed in Blanden, 1974; McMichael *et al.*, 1983; Mims and White, 1984a). In retrospect, the relative importance of cellular versus humoral immunity in the human primary response to poxviruses (and viruses in general) was evident in the observations of patients with immune deficiency diseases (Fulginiti *et al.*, 1968). Patients with congenital or acquired aggammaglobulinemia could be successfully vaccinated against smallpox and recovered from measles and other childhood viral diseases. In contrast, thymus-deficient children developed severe generalized vaccinial infections following vaccination and were not helped by transfusion of immune globulin. Such observations could not be interpreted fully prior to the definition of lymphocyte subsets and their functions.

Among the most definitive demonstrations of the antiviral effector functions of Tc cells are the models of host defense to primary viral infections in mice (Blanden, 1971b; Zinkernagel and Welsh, 1976; Yap and Ada, 1978b; Yap et al., 1978; Reddhase *et al.*, 1987).

In a series of crucial experiments, Blanden (1970; 1971a, b) established an essential role for T cells in recovery from mousepox, a generalised viral infection in mice. An important event in the recovery process was the recruitment of mononuclear phagocytes into foci of infection by specifically activated antiviral Tc cells. Based on these studies, a mechanism whereby Tc cells exerted antiviral function *in vivo* was suggested (Blanden, 1971b). Firstly, by virtue of their cytolytic capability, antiviral Tc could halt virus spread by destruction of infected cells before assembly of progeny virus. Secondly, the recruitment of mononuclear phagocytes by the antiviral Tc cells and their subsequent activation would allow the destruction of virus-infected material by phagocytosis. Finally, effector Tc cells may secrete IFN at sites of infection, thus protecting other cells in the immediate vicinity from being infected. Subsequent studies by Blanden and Gardner (1976) indicated that the kinetics of generation of splenic anti-ectromelia virus Tc cell activity were similar to transferred protective activity. They raised the possibility that the effector T cell populations that lysed virus-infected targets *in vitro* could be identical to those which conferred protection *in vivo*.

The *in vivo* protective effect of antiviral Tc cells is absolutely dependent on the transferred immune Tc cells and the recipient sharing at least the *K* or *D* regions of the *H-2* gene complex, but *H-2I* region-compatible T cells do not confer any significant level of protection (Kees and Blanden, 1976; Zinkernagel and Welsh, 1976; Ertl *et al.*, 1977; reviewed in Zinkernagel and Doherty, 1979). Using H-2 mutant mice, the fine specificity of the MHC restriction of T-cell mediated antiviral protection was shown to correspond to that of Tc cells (Kees and Blanden, 1976).

The direct involvement of Tc cells in antiviral immunity through their ability to destroy infected host cells is strongly suggested by numerous *in vitro* and *in vivo* experimental systems (Blanden and Gardner, 1976; Ada *et al.*, 1976; Zinkernagel and Althage, 1977; Koszinowski and Ertl, 1976; Zinkernagel *et al.*, 1977; Yap and Ada, 1978a, b; Yap *et al.*, 1978; reviewed in Zinkernagel and Doherty, 1979; Lukacher *et al.*, 1984). Mullbacher and Ada (1987) have discussed evidence in favour of the cytolytic function of Tc cells *in vivo* but there is no direct evidence to demonstrate the *in vivo* killing of virus-infected cells in solid tissues. Recently, Liu and Mullbacher (1989) demonstrated that transferred alloreactive Tc cells kill host macrophages in the peritoneal cavity by programmed cell death (apoptosis), one mode of killing exercised by Tc cells. Thus, it may be possible to demonstrate antiviral Tc cell- mediated lysis of infected cells by apoptosis in solid tissues. It should be emphasised that Tc cell-mediated lysis can occur by a number of ways and apoptosis is only one of them. The question of whether Tc cells exert antiviral function *in vivo* through cytolytic activity still remains controversial and will not discussed further. What warrants consideration, however, is the the role of cytokines, particularly IFN-γ, in Tc cell-mediated antiviral function *in vivo*.

Both alloreactive and antiviral $CD8^+$ Tc cells produce IFN-γ when stimulated appropriately in an antigen-specific and MHC-restricted manner (Glasebrook *et al.*, 1981; Morris *et al.*, 1982; Klein *et al.*, 1982; Prystowsky *et al.*, 1982; reviewed in Mosmann and Coffman, 1989). The availability of cyclosporine A (CsA) which inhibits the synthesis and secretion of cytokines like IL-2 and IFN-γ by Tc cells but not the cytotoxic activity (Andrus and Lafferty, 1982; Prowse *et al.*, 1985; Schiltknecht and Ada, 1985a, b, c; Hodgkin *et al.*, 1987; Noble and Steinmuller, 1989;

Granelli-Piperno, 1990) has provided an excellent tool for the analysis of the physiological function of Tc cells *in vivo*.

In a series of experiments, Schiltknecht and Ada (1985a, b, c) established that in CsA-treated mice, influenza virus could not be cleared and persisted at very high titres; specific immune responses were delayed or of lesser magnitude (antibody and Tc cell responses) or apparently absent (DTH response), compared to untreated controls (Schiltknecht and Ada, 1985a). $CD4^+$ T cells obtained from CsA-treated, virus-infected mice could transfer DTH to recipient mice infected a few hours earlier with influenza virus, suggesting that anti-influenza Td cells were generated in mice treated with CsA but the effectors could not function *in vivo* (1985b). Similarly, in adoptive transfer experiments with anti-influenza Tc cells, transferred cells did not reduce virus titres in CsA-treated animals, unlike untreated controls (Schiltnecht and Ada, 1985c). Equal numbers of the transferred cells were shown to reach lungs in both groups. These findings led them to suggest a role for lymphokine release by Tc *in vivo* for antiviral protection. Their suggestions were consistent with an earlier report by Taylor and Askonas (1983) who described the *in vivo* antiviral functions of two influenza virus-specific T-cell clones. Only the clone that produced IFN-γ on exposure to influenza virus-infected syngeneic targets *in vitro* reduced lung virus titres *in vivo*. This was despite the fact that a smaller number of cells of the IFN-γ-producing clone reached the lungs compared to the other clone. However, data obtained by yet another group (Lukacher *et al.*, 1984) suggested that it was the direct cytolytic activity and not lymphokine production which was critical for the expression of antiviral function by Tc cells in the influenza model. Based on these findings, Schiltknecht and Ada (1985c) postulated that that the lymphokines secreted by T cells *in vivo* may have an indirect role. The secretion of IFN-γ by Tc cells could enhance the expression of class I MHC antigens on infected lung cells which would make them more susceptible to lysis.

The relative importance of IFN-γ-induced upregulation of MHC antigens for the expression of Tc cell function *in vivo* is exemplified by a recent study using alloreactive Tc cells. Lafferty and colleagues (Prowse *et al.*, 1985) previously established that islet graft destruction by primed $CD8^+$ T cells is CsA sensitive, and therefore islet rejection is a cytokine-dependent process. To further address the role of cytokines in Tc cell-mediated graft

rejection, they (Hao *et al.*, 1990) used an alloreactive $CD8^+$ Tc cell clone which produced IFN-γ and TNF-β but not IL-2 or IL-3. The clone, known to transfer allograft immunity was found to be sensitive to CsA *in vivo*, suggesting that IFN-γ and/or TNF-β may be involved in allograft rejection. Thus, mice transplanted with pancreatic islets rejected the graft when the alloreactive Tc cell clone was transferred but failed to do so if treated with CsA. However, pretreatment of the islet tissue with IFN-γ prior to grafting was shown to increase class I MHC antigen expression on the islet tissue and CsA could no longer block the destruction of these islets *in vivo* by the transferred alloreactive Tc cells in 11 of 15 mice. The authors concluded that the cytokine dependence of islet allograft rejection was due, at least in part, to the activity of IFN-γ which acts by increasing MHC antigen density on the target tissue, rendering it sensitive to a cytotoxic attack. This finding is consistent with the postulation of Schiltknecht and Ada (1985c) but the possibility that IFN-γ may also contribute to allograft rejection or antiviral function by other means cannot be excluded. Other potential roles of IFN-γ in antiviral immunity will be addressed further in Chapters 5.

A recent study (Ruby and Ramshaw, submitted) has provided evidence that anti-VV $CD8^+$ Tc cell function *in vivo* is dependent on IFN-γ. Normal CBA/H mice treated daily with a Mab to IFN-γ following infection with VV were unable to clear virus, which resulted in 100% mortality from an otherwise sublethal infection. In another VV-infection model, transfer of $CD8^+$, anti-VV Tc cells to infected mice reduced VV titres by 4 $\log_{10}$ PFU within 3 days. However, the antiviral activity of transferred anti-VV Tc cells was completely abrogated in recipient mice which had been infected with VV and treated with anti-IFN-γ Mab, indicating that IFN-γ was a crucial component of Tc cell-mediated clearance of VV.

The importance of Tc cells in control of viral infections has been further demonstrated using athymic nude mice. Due to a lack of functional T cells, these mice usually succumb to viral infections and die (Ertl *et al.*, 1977; Grundy and Melief, 1982; Yap *et al.*, 1979). Influenza virus-infected nude mice can be saved by transfer of immune Tc cells (Yap *et al.*, 1979) but not immune serum (Yap and Ada, 1979). Recently, Ramshaw and colleagues (1987) have shown that such T-cell deficient mice can successfully resolve an infection with recombinant VV encoding murine IL-2. Further investigations revealed that the recovery was not T-cell

mediated and suggested a possible involvement of NK cells (Karupiah *et al.*, 1990) and IFN-γ. The mechanisms of recovery of nude mice from infection with the IL-2-encoding VV will be addressed in the later chapters.

As discussed, classical $CD8^+$ antiviral Tc cells only recognize epitopes consisting of a complex of classI MHC with a peptide derived from viral antigen expressed in the cytosol (Townsend *et al.*, 1985; Townsend *et al.*, 1986a, b; Morrison *et al.*, 1986a, b; Braciale *et al.*, 1987a; reviewed in Townsend and Bodmer, 1989). Because these class I molecules are expressed virtually on all somatic cells, any infected cell can be eliminated by the appropriate virus-specific Tc cells. However, MHC class II-restricted $CD4^+$ Tc cells do exist and their possible physiological role is discussed below.

3.2.5.2. $CD4^+$ MHC class II-restricted Tc cells

The existence of class II MHC-restricted, $CD4^+$ Tc cell responses have been reported for influenza virus (Fleischer *et al.*, 1985; Lukacher *et al.*, 1985; Morrison *et al.*, 1986a, b), HSV (Schmid, 1988; Yasuka and Zarling, 1984), EBV (Meuer *et al.*, 1983; Misko *et al.*, 1984) and measles virus (Jacobson *et al.*, 1984). Whereas influenza virus infection elicits both class I- and class II-restricted Tc, the Tc response to measles virus is primarily class II- restricted. In all of the above studies, cloned $CD4^+$ Tc cells have been used. A cloned anti-influenza $CD4^+$, class II-restricted line with Tc activity was even shown to provide protection in mice against lethal influenza infection (Lukacher *et al.*, 1985). While the above finding provides definitive evidence for an antiviral function, the relative importance of class II-restricted Tc in comparison to class I-restricted Tc cells during a normal (or experimental) infection would vary in different virus-host combinations and different target organs. From the studies of Lukacher *et al* (1985) it is not clear how class II-restricted Tc cells would directly eliminate virus-infected cells that only express class I MHC antigens.

3.2.6. Helper T (Th) cells

$CD4^+$ T cells are generally designated helper (Th) cells but this terminology is inappropriate. This subset of T cells mediates a variety of different functions, one of which is to provide 'help' to antigen-specific B cells and $CD8^+$ Tc cells for activation. It is now evident that $CD4^+$ T cell

help is not mandatory for the generation of $CD8^+$ antiviral Tc cell responses. The 'help' provided by $CD4^+$ T cells is through secretion of cytokines involved in lymphocyte activation. It is also clear that $CD8^+$ T cells can produce cytokines and hence provide helper functions for the generation of Tc cell responses (Andrus *et al.*, 1984; Mizuochi *et al.*, 1985, 1989).

Since most viral antigens are T-dependent (Burns *et al.*, 1975), the generation of virus-specific immune responses requires interaction between $CD4^+$ Th cells and APCs. $CD4^+$ Th cells recognize an epitope formed by a complex of viral peptide on APCs with class II MHC molecule (Unanue and Allen, 1987; Unanue and Cerottini, 1989). The TCR binding to this epitope initiates activation of $CD4^+$ Th cells followed by diverse cellular interactions that result in antigen-specific B cell activation and development of inflammatory reactions.

B-cell activation is induced by cytokines secreted by $CD4^+$ Th cells. These cytokines, namely IL-4, IL-5 and IL-6 are involved in B-cell growth and differentiation and antibody isotype switching (reviewed in Kishimoto, 1985; O'Garra *et al.*, 1987; Kishimoto, 1988; reviewed in Mosmann and Coffman, 1989). IL-4 and IL-5 are only produced by $CD4^+$ T cells of the Th2 subset which also utilize IL-4 as an autocrine growth factor. $CD4^+$ Th1 cell-derived cytokines such as IL-2 and IFN-γ are also involved in antigen-specific B cell responses. T cell help is essential for the generation of antigen-specific IgA and IgG antibody responses. The role of antibody in antiviral responses will be briefly dealt with in section 3.3.

Inflammatory responses are mediated by $CD4^+$ T cells of the Th1 subset which secrete IL-2 and IFN-γ and utilize IL-2 as an autocrine growth factor (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989; Bottomly, 1988). Some of these clones lyse antigen-specific, class II MHC-restricted targets (Bottomly, 1988). The role of $CD4^+$ inflammatory T (Td) cells in antiviral immunity will be discussed in section 3.2.7.

Reports on the requirements for $CD4^+$ T cell help in the generation of antiviral Tc cells are controversial. A number of *in vitro* and *in vivo* studies suggest that T cell help is necessary for the generation of antiviral Tc cell responses to poxviruses, influenza virus, Sendai virus (SV) and VSV (Pang *et al.*, 1976; Ashman and Mullbacher, 1979; Brenan and Mullbacher, 1981; Ciavarra, 1990). From experiments with radiation

chimaeras, T cells differentiating in an H-2-compatible, but H-2I-incompatible thymic environment were found to be completely unresponsive to VV (Zinkernagel *et al.*, 1978). This was interpreted to mean that participation of Th cells was a necessary requirement for anti-VV Tc cell generation and hence the need for I-region compatibility. These views were not favoured by Bennink and Doherty (1978), who, based on their own data, suggested that T cell help was not required for the generation of anti-VV Tc cell response.

Studies performed using Mab to selectively deplete $CD4^+$ T cells *in vivo* have provided but somewhat conflicting findings. In one such study, Buller *et al* (1987) demonstrated that $CD4^+$ T cell depletion did not affect the Tc cell response to ectromelia and VV. Furthermore, $CD4^+$ T cell-depleted mice recovered from infection with ectromelia virus which was lethal for athymic nude mice. Using similar approaches, $CD4^+$ T cells were found to be unnecessary for the generation of antiviral Tc cell responses to HSV (Nash *et al.*, 1987), LCMV (Ahmed *et al.*, 1987) and influenza virus (Liu and Mullbacher, 1989). In the case of HSV, depletion of $CD4^+$ T cells was, in fact, found to enhance the anti-HSV Tc cell response (Nash *et al.* 1987).

Two other recent reports on the role of $CD4^+$ T cells in antiviral Tc cell generation suggest that Th function may be important. $CD4^+$ T cell depletion was shown to impair Tc cell responses to Moloney Sarcoma virus (MSV) (Weyland *et al.*, 1989), LCMV and VV (Leist *et al.*, 1989). While the reasons for differences between these (Leist *et al.*, 1989) and the earlier reports (Buller *et al.*, 1987; Ahmed *et al.*, 1987) in the case of Tc cell responses to VV and LCMV are not clear, it is interesting to note that administration of exogenous IL-2 to $CD4^+$ T cell-depleted mice either partially (Leist *et al.*, 1989) or completely (Weyland *et al.*,1989) restored the antiviral Tc cell responses. These findings are consistent with the idea that 'help' provided by $CD4^+$ Th cells is in the form of IL-2. Indeed, in the absence of $CD4^+$ Th cells, IL-2-producing $CD8^+$ T cells were found to be sufficient for the optimal generation of anti-VV Tc cell response (Mizuochi *et al.*, 1989). It is not clear whether IL-2 is the only factor necessary. In this regard, Liu and Mullbacher (1989) found that in the absence of $CD4^+$ T-cells, 'help' could be delivered *in vitro* by B cells with no requirement for cell to cell contact and in the apparent absence of IL-2 or IL-4, but the precise mechanisms are not known. Furthermore, a

recent report by Ciavarra (1990) indicates that the generation of a secondary VSV Tc cell response *in vitro* in the absence of $CD4^+$ T cells could not be replaced by addition of exogenous IL-2 to the culture. Addition of IL-2 was reported to induce cytotoxic cells which had the characteristics of LAK cells but not antiviral Tc cells.

Clearly, the above findings demonstrate that the contribution of $CD4^+$ T cells in the induction of antiviral Tc cell responses was not always mandatory and may vary in different virus infection models, suggesting that $CD4^+$ T cells may not be crucial for the expression of antiviral function by $CD8^+$ Tc cells. There is strong evidence (Nash *et al.*, 1987), however, that $CD4^+$ T cells mediating DTH (Td) are necessary for the control of HSV infection in skin even in the presence of $CD8^+$ Tc cells (see below).

3.2.7. T cells and delayed type hypersensitivity (Td)

Delayed type hypersensitivity (DTH) is a T cell-mediated inflammatory reaction characterized by mononuclear cell infiltration. In response to a viral infection, virus-specific T cells proliferate and release cytokines which attract macrophages to the site of viral replication and in turn induce them to proliferate (Liew, 1982). The classical T cells (Td) that mediate DTH are $CD4^+$ and hence class II MHC-restricted. However, DTH is not exclusively a $CD4^+$ T cell-mediated function. Several lines of evidence indicate that class I MHC-restricted $CD8^+$ T cells can mediate DTH functions (Zinkernagel, 1976; Ada *et al.*, 1981; Lin and Askonas, 1981).

Recent studies using cloned $CD4^+$ Th cells and $CD8^+$ alloreactive Tc cells provide a basis for understanding why both $CD4^+$ and $CD8^+$ T cells are capable of mediating DTH reactions (Mosmann *et al.*, 1986; Bottomly, 1988; Mosmann and Coffman, 1989). Based on distinct cytokine secretion patterns, cloned $CD4^+$ Th cells may be divided into two subsets. Th1 clones secrete IL-2 and IFN-γ but not IL-4 or IL-5, and mediate DTH functions. Th2 clones, on the other hand, secrete IL-4 and IL-5 but not IL-2 or IFN-γ and provide help for B cell responses. Furthermore, Th1 clones but not Th2 clones, produce TNF-β and lyse targets in an antigen-specific and MHC-restricted manner (Bottomly, 1988). Additionally, some alloreactive $CD8^+$ Tc cell clones have a cytokine secretion pattern similar to that of Th1 clones. One of the cytokines produced by Th1 clones and

alloreactive $CD8^+$ Tc cell clones, IFN-γ, has been implicated as an essential mediator of DTH and in the migration of leukocytes into inflammatory sites (Issekutz *et al.*, 1988). Hence, it is conceivable that both $CD4^+$ and $CD8^+$ T cells may mediate DTH reactions in a viral infection. However, the crucial question concerns the importance of such a response in antiviral immunity. Is DTH essential for the control of a viral infection? Perhaps the following examples may illustrate its relative importance.

Leung and Ada (1981) showed that class I MHC-restricted $CD8^+$ T cells that included cells with Tc and Td activity, could protect mice against lethal influenza virus infection whereas class II MHC-restricted $CD4^+$ T cells with Th and Td activity exacerbated the disease and accelerated the death of mice. Transfer of $CD4^+$ T cells with DTH activity but undetectable Tc activity was not protective (Liew and Russel, 1983), while transfer of $CD4^+$ T cells with Tc activity provided protection in mice against lethal influenza infection (Lukacher *et al.*, 1985). . Both $CD4^+$ and $CD8^+$ T cells appear to mediate DTH activity (Leung and Ada, 1981) but the Tc component may be more important for influenza virus clearance *in vivo*. Furthermore, a cloned influenza HA-specific, class I-restricted Tc cell line, (Lin and Askonas, 1980) that conferred protection in mice following adoptive transfer *in vivo*, and had the capacity to lyse influenza-infected targets *in vitro*, was subsequently shown to confer DTH skin reactivity to influenza virus (Lin and Askonas, 1981). The same clone also produced IFN-γ after exposure to influenza virus-infected cells *in vitro* (Morris *et al.*, 1982). Based on the other reports (Leung and Ada, 1981; Lukacher *et al.*, 1985) it is tempting to speculate that the Tc component was probably more important but the possibility exists that both functions (cytotoxicity and DTH) of the clone contributed to antiviral mechanisms. Also, although IFN-γ is part of the DTH component, the possibility that this lymphokine contributed to direct antiviral activity and increased the efficiency of cytotoxic function cannot be excluded.

In the studies performed to demonstrate that antiviral Tc cell induction and generation do not require the participation of $CD4^+$ Th cell (Buller *et al.*, 1987; Nash *et al.*,1987; Ahmed *et al.*, 1987; Liu and Mullbacher, 1989), few have reported the pathological findings or data on viral titres. The relative importance of $CD4^+$ T cells in the process of recovery from a viral infection is exemplified by the report of Nash *et al* (1987) which

demonstrated that $CD4^+$ T cells were not mandatory for the generation of anti-HSV Tc cell response. But the depletion of $CD4^+$ T cells in mice resulted in a delay in clearance of HSV from the skin despite the presence of $CD8^+$ antiviral Tc cells and such mice exhibited increased mortality. Additionally, in mice depleted of $CD8^+$ T cells but intact $CD4^+$ T cells (hence DTH response), virus clearance from the ear was normal whereas elimination of virus from the peripheral and central nervous system was markedly delayed although no increase in mortality compared to control mice was observed. Thus, in the HSV model, both $CD4^+$ and $CD8^+$ T cells are important and contribute to viral clearance at different sites of viral replication. From the studies conducted by Buller *et al* (1987), it is evident that depletion of $CD4^+$ T cells did not result in death after ectromelia virus infection, but the necrotic lesions in spleen, liver and lymph nodes of $CD4^+$ T-cell-depleted mice were clearly more severe. This is suggestive of a role for $CD4^+$ T cells in limiting viral growth, perhaps in some indirect manner. Efficient viral clearance seems to be a function of $CD8^+$ Tc cells since depletion of $CD8^+$ T cells in mice (although achieved with difficulty) resulted in death after infection with ectromelia virus (Buller *et al.*, 1987).

Although Tc cells are important in the process of recovery from most primary virus infections (Blanden, 1974), they appear only to halt further virus spread, and cannot reduce the number of infectious particles already present as was demonstrated for ectromelia (Blanden, 1971b). The beneficial effect of Tc cell-mediated lysis is apparent only if infected cells are lysed before assembly of progeny virus. Therefore, the advantage of a DTH reaction is the recruitment of monocytes. If infectious virus were released from infected cells in solid tissues before the generation of neutralising antibody or in sites where antibody did not readily penetrate, then recruitment of mononuclear phagocytes, which phagocytose and destroy infectious material or become unproductively infected, would surely help. The importance of DTH reactions in the process of recovery from viral infections has been previously questioned (Liew, 1982), but it is apparent that the DTH reaction contributes to antiviral mechanisms in some infection models. The extent to which it contributes to viral clearance from infected tissues may vary with different viruses in different tissues of different host species.

3.3. Humoral responses in antiviral immunity

Although T-cell-mediated immunity (CMI) plays a crucial role in the recovery from most primary viral infections, including poxviruses, influenza, herpes-type viruses, CMV, LCMV and measles, an appropriate antibody response is important in the recovery from some viral infections like yellow fever, polio and coxsackie (Mims, 1987a). Humoral responses are particularly important in viral infections that produce systemic disease with plasma viraemia. However, virus-specific antibody is the first line of defense to reinfection (Oldstone, 1975; Mims and White, 1984c; Mims, 1987a).

The generation of CMI precedes humoral responses in most primary viral infections including poxviruses (Blanden, 1970; Hutt 1975; Pyne, 1980; Novembre *et al.*, 1989; Mims and White, 1984a; Mims, 1987a), but virus-specific antibody could contribute to the termination of infection, particularly in the clearance of plasma-borne virus. Virus neutralisation can occur by direct antibody binding and in the process prevent infection of susceptible cells in a number of ways (Della-Porta and Westaway, 1978; Possee *et al.*, 1982; Wiley *et al.*, 1981; Taylor and Dimmock, 1985a, b). Control of infection can also occur by ADCC and activation of the classical complement pathway which is antibody-dependent (MacFarlan *et al.*, 1977; Shore *et al.*,1976; Rager-Zisman and Bloom, 1974; reviewed in Hirsch, 1982). Antibody-mediated immunity may operate via all the above mechanisms during a primary or secondary infection.

4. Recombinant VV: Live virus vaccines and analysis of the immune response to defined foreign antigens

4.1. General

Smallpox was among the first diseases shown amenable to prophylaxis, the means of prevention having apparently been discovered long before the elaboration of the germ theory of infectious disease. Now rendered extinct, smallpox has been known for centuries as one of mankind's most serious scourges (Fenner, 1985; Fenner *et al.*, 1988a). The eradication of smallpox on a global scale was made possible by the extensive use of vaccinia virus (VV) vaccines (reviewed in Behbehani, 1983). Routine vaccination was stopped in 1980 on the recommendations of the World Health Organization.

4.2. Recombinant VV as live virus vaccines

Within two years of the discontinuation of smallpox vaccination, the prospect of live vaccines consisting of genetically engineered vaccinia viruses encoding foreign antigens was reported (Mackett *et al.*, 1982; Panicali and Paoletti, 1982). Following these first reports, a large number of VV recombinants encoding viral, bacterial,protozoan and human proteins have been constructed (reviewed in Mackett and Smith, 1986; Moss, 1985; reviewed in Moss and Flexner, 1987) and analysed in terms of correct synthesis, processing and transport of proteins. This approach also has allowed the analysis of immune responses to single defined antigens and further to define "protective antigens" from pathogens.

4.3. Protection

A number of studies utilizing VV recombinants have provided strong evidence that the co-expressed foreign antigens can prime the immune system and provide protection in mice, rats, foxes, hamsters, cattle and chimpanzees against the appropriate pathogens (Smith *et al.*, 1983; Kieny *et al.*, 1984; Moss *et al.*, 1984; Mackett *et al.*, 1985; Blancau *et al.*, 1985; reviewed in Mackett and Smith, 1986; Andrew *et al.*, 1987; Elango *et al.*, 1987; Koprowski *et al.*, 1987; reviewed in Flexner and Moss, 1987; Bray et al., 1989).

4.4. On the question of safety

While the potential for developing this approach to vaccination is enormous, no compromise can be made on the question of safety. Post-vaccination complications, both severe and mild, following immunization with VV have been well documented (Fenner *et al.*, 1988b). This has been despite the availability of information relating to the safety of virus strains used during the WHO smallpox eradication program

Notable in this regard is the demonstration by Buller *et al.* (1985) that deletion of the thymidine kinase (TK) gene of the WR strain of VV effectively reduced virulence but did not affect immunogenicity. In contrast, evidence from the work of Andrew and colleagues (1989) has indicated that recombinant VV with a TK^- phenotype induced lower antibody and Tc cell responses than the wild type (TK^+ phenotype) VV, which replicated to higher titres. While the safety of a vaccine is of high priority, reduction of its virulence may also result in reduced immunogenicity which is undesirable.

A rather different approach was taken by Ramshaw and colleagues (1987). They inserted the murine IL-2 gene into a recombinant VV genome, which also contained the gene for influenza HA, in order to enhance the immune response to VV and the co-expressed foreign antigen. They reported the interesting finding that athymic, nude mice that lacked functional T cells successfully cleared infection with the IL-2-encoding virus, but died of disseminated vaccinial disease after infection with a control recombinant virus. Soon after this, Flexner *et al* (1987) showed that insertion of the human IL-2 gene into VV genome led to nude mice surviving the infection. The role of antiviral Tc cells in mediating recovery from primary poxvirus infections is well established (Hirsch *et al.*, 1968; Blanden, 1971b; Ertl *et al.*, 1977). Therefore, the finding that expression of murine or human IL-2 during an infection with the recombinant VV allows the survival of T-cell deficient mice (Ramshaw *et al.*, 1987; Flexner *et al.*, 1987), was intriguing. Investigation of this and related phenomena is reported in this thesis.

5. Scope of this thesis

The demonstration by Ramshaw *et al.* (1987) that T-cell deficient nude mice recover from an infection with recombinant VV encoding murine IL-2 had important implications and therefore required a detailed analysis of the mechanisms involved.

The experiments described in this thesis were designed to study the immunobiology of infection with the IL-2-encoding VV, designated VV-HA-IL2, with particular emphasis on the recovery mechanisms in nude mice.

In Chapter 2 , the kinetics of recombinant VV replication in athymic, nude and euthymic normal mice are described. Studies on the survival of nude mice following infection with VV-HA-IL2 and a control recombinant not encoding IL-2 (VV-HA-TK) have been included. Data are presented which show that VV-HA-IL2 is cleared more rapidly than VV-HA-TK in nude and normal mice and that clearance is not likely to be antibody-mediated.

Chapter 3 examines the possible roles of NK and Tc cells in rapid clearance of VV-HA-IL2. The kinetics of viral clearance precede that of Tc cell generation in normal mice but coincides with an elevated NK cell response in both nude and normal mice infected with VV-HA-IL2. Nude mice infected with VV-HA-IL2 had no detectable antiviral Tc cell response.

In view of these findings, in Chapter 4, the possible role of NK cells is addressed using NK cell-deficient mouse models. The results were suggestive of both NK-dependent and -independent mechanisms involved in the rapid clearance of VV-HA-IL2 and survival of nude mice.

Chapter 5 documents investigations into the role of soluble mediators with antiviral function that could be triggered by virus-encoded IL-2 and that may have contributed to the recovery of nude mice from infection with VV-HA-IL2. The approach taken was the use of monoclonal antibodies *in vivo* to inhibit the function of the soluble mediators. A crucial role for IFN-γ was established and evidence is presented indicating that IFN-γ-mediated macrophage activation may be involved in the recovery mechanisms.

Chapter 6 has been included to illustrate pertinent observations made in the course of the work described above which relate to the highly productive infection that VV causes in murine ovaries. I have addressed these observations with regard to virus virulence, viral tropism and how the expression of IL-2 during an infection with VV-HA-IL2 can avert ovarian damage and prevent infertility in mice.

Finally, in Chapter 7 I have attempted to evaluate the data described in this thesis.

CHAPTER 2

Studies on the Kinetics of Recombinant VV Replication and Clearance in Nude and Normal Mice

INTRODUCTION

Ramshaw and colleagues (1987) reported that athymic nude mice infected with the IL-2-encoding recombinant virus, VV-HA-IL2, resolved the infection successfully whereas nude mice given the control virus, VV-HA-TK, developed a progressive vaccinial disease and died. VV-HA-IL-2 given i.v. or s.c. in the foot-pad to nude mice was cleared by 11 and 15 days respectively. VV-HA-TK given in the foot-pad of nude mice induced a severe necrotic lesion which remained unresolved even 30 days post-infection. All nude mice given VV-HA-TK i.v. died within 15 days after infection.

In order to study the IL-2-induced mechanism(s) of recovery of nude mice from VV-HA-IL2 infection and the immunobiology of infection with the recombinant virus, an investigation of the kinetics of viral replication and clearance in athymic nude was an essential starting point. Although the rates of VV-HA-TK and VV-HA-IL2 clearance from foot-pads of normal euthymic CBA/H mice has been shown previously (Ramshaw *et al.*, 1987) to be comparable, viral replication/clearance in the organs of these immunocompetent mice required investigation.

This chapter descibes studies on the kinetics of replication and clearance of the recombinant vaccinia viruses in nude and normal mice. Mortality in nude mice infected with different doses of the wild type virus, VV-WR, and the recombinants VV-HA-TK and VV-HA-IL2 has also been included. In addition, experiments were carried out to establish that IL-2 was produced *in vivo* during an infection with VV-HA-IL2.

MATERIALS AND METHODS

Mice.

Mice raised under specific pathogen-free conditions were used at 4-10 weeks of age. Athymic nude (nu/nu) mice of the CBA background were obtained from the Walter and Eliza Hall Institute, Melbourne. Euthymic CBA/H and athymic Swiss outbred nude mice were obtained from the Animal Services Section of the John Curtin School of Medical Research.

Viruses.

A brief description of the recombinant VV used throughout this study is necessary to understand the work described in this chapter and the proceeding chapters. The neurovirulent, L929 cell-adapted WR strain, (Wokatsch, 1972), designated VV-WR, was used to construct the recombinants (Ramshaw *et al.*, 1987). The murine IL-2 encoding recombinant, VV-HA-IL2, and the control virus VV-HA-TK, were both constructed from a previously described recombinant VV-PR8-HA6 (Andrew *et al.*, 1986), which encodes the haemagglutinin (HA) of influenza A/PR/8/34 virus. VV-PR8-HA6 will be referred to as VV-HA.

A *Hind*III map of VV-WR is shown in Fig. 1a with insertion points at *Eco* RI (E) and *Bam*HI (B) sites in the J and F regions respectively (Fig. 1b). The influenza HA gene, under the control of the vaccinia 7.5-kD promoter ($P_{7.5}$), was inserted into the vaccinia TK gene in the J region at the *Eco*RI site (Fig. 1c) since this allowed the selection of recombinant virus by virtue of its TK^- phenotype (Mackett *et al.*, 1982). VV-HA-IL2 was constructed by insertion of the HSV TK gene plus a chimaeric promoter-IL-2 fragment into the *Hind*III F region at the *Bam*HI site of VV-HA (Fig. 1e). The insertion of HSV TK, under the control of a promoter (P_F) derived from the the *Hind*III F fragment of VV DNA, and the IL-2 gene, under the control of $P_{7.5}$, resulted in a TK^+ phenotype. Since the HSV TK gene was also used as a selectable marker for virus construction, the control recombinant virus VV-HA-TK encoding HSV TK but not IL-2 was similarly constructed (Fig. 1d). The genome arrangements of the recombinant viruses were analysed by restriction endonuclease digestion and hybridization with the appropriate DNA probes (Ramshaw *et al.*, 1987). *In vitro*, both recombinant viruses had comparable rates of replication. High levels of biologically active IL-2 were detected in the

supernatants of VV-HA-IL2-infected murine, primate and human cells but not with VV-HA-TK.

Virus stocks were prepared from infected CV-1 cells [(0.01 plaque forming units (PFU)/cell] grown in 2 litre Schott-acid washed roller bottles. Titrated stocks were stored in 100µl aliquots in sterile vials at -70 °C. Before use for immunization, virus stocks were diluted in 2 ml of gelatin saline to contain the desired PFU and sonicated at 50 watts for 2x5 seconds using a Branson Sonifier (Branson Sonic Power Company, Danbury, CT., USA).

Cell lines.

CV-1, a cell line derived from African green monkey kidney (Jensen *et al.*, 1964) and 143B, a human osteosarcoma cell line (Rhim *et al.*, 1975) were maintained in Eagle's minimum essential medium (EMEM) (Gibco, Grand Island, NY, USA) supplemented with antibiotics and 5% heat inactivated (56°C for 30 minutes) foetal calf serum (FCS) (Flow Laboratories, North Ryde, Australia).

Virus titration.

Organs removed from mice infected with VV-HA-TK or VV-HA-IL2 were stored individually in 1 ml of phosphate buffered saline (PBS) at -70 °C. Organs from individual mice were homogenized and a 100 µl volume of the homogenate was incubated with an equal volume of trypsin (1 mg/ml) in gelatin saline for 30 min at 37°C. After neutralization of the trypsin with 800 µl of saline containing 5% FCS, serial 10-fold dilutions were made and the virus titrated as plaques grown on human 143B cell monolayers in 6 well Linbro plates (Flow Laboratories, Inc, McLean, VA). Virus was adsorbed for 1 h before the cell monolayers were overlaid with EMEM containing 2.5% FCS. After a 2 day incubation period at 37 °C in a humidified atmosphere containing 5% CO_2 in air, monolayers were fixed and stained with 0.1% crystal violet in 20% ethanol for 5 min, dried and plaques counted.

Assay for IL-2.

The presence or absence of murine IL-2 in cell culture supernatants and sera of mice infected with VV-HA-IL2 or VV-HA-TK was determined using the murine IL-2-dependent T-cell line CTL-L. The procedure has been described in detail elsewhere (Gillis *et al.*, 1978). Briefly, presence of IL-2 was assessed by CTL-L cell proliferation and uptake of ^{3}H-thymidine in the presence of serial 2-fold or 3-fold dilutions of sera.

Monoclonal antibody (Mab) to murine IL-2

A rat anti-mouse hybridoma line, S4B6.31, which is a subclone of the originally described clone, S4B6 (Mosmann *et al.*, 1986), was obtained from DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA., USA. The clone secretes Mab (IgG_{2a} subclass) to murine IL-2. The Mab was obtained as ascites, grown in pristane-primed outbred nude mice and used after partial purification by ammonium sulphate precipitation. The precipitate was dissolved in PBS and dialysed 3 times against PBS at 4^{o}C , filter sterilized and stored at -20 ^{o}C. This Mab was used to neutralize the activity of IL-2 *in vivo* as described in Results.

RESULTS

Mortality in outbred nude mice given VV-WR or VV-HA-TK but not VV-HA-IL2.

Groups of female outbred nude mice were injected i.v. with varying doses of the wild type virus VV-WR or the recombinant viruses, VV-HA-TK and VV-HA-IL2. Mice that received 10^6 or 10^7 PFU of VV-WR developed severe pox lesions on the skin between days 3 and 6, exhibited weight loss (data not shown) and weakness and died between 6 and 10 days after infection (Table 1). The mean time to death (MTD) in nude mice given VV-HA-TK was dose-dependent in that mice given $5x10^5$ PFU of virus had a MTD of 23 days (Table 1) whereas mice given 10^7 PFU of virus had a MTD of 14.8 days ($p< 0.01$, Students T-test). The wild type virus appeared more virulent than the control recombinant virus since nude mice that received VV-WR developed disease signs and died earlier than mice that had received the same dose of VV-HA-TK (Table 1). Nevertheless, infection with VV-WR or VV-HA-TK in outbred nude mice resulted in 100% mortality. In contrast, all mice that had been infected with either 10^7 or $5x10^7$ PFU of VV-HA-IL2 appeared healthy with no signs of disease over the entire observation period of 21 days.

Mortality in inbred CBA nude mice.

The observations made with outbred nude mice were consistent with those seen in inbred CBA nude mice. All of the CBA nude mice that were inoculated i.v. with 10^7 PFU of VV-HA-IL2 survived the infection with no overt disease. There was 100% mortality in mice given 10^6 or 10^7 PFU of VV-HA-TK with MTD of 13.6 and 11.2 days respectively (Fig. 2).

Kinetics of recombinant VV replication in organs of nude mice

Female outbred nude mice were infected i.v. with 10^7 PFU of VV-HA-TK or VV-HA-IL2 and the kinetics of viral replication and clearance in organs of mice was determined over a 13 day period.

In ovaries of infected mice, VV-HA-TK and VV-HA-IL2 titres were comparable 0.5, 1 and 2 days after infection (Fig. 3). Both recombinant viruses replicated very rapidly in ovaries with mean virus titres increasing from 3 $\log_{10}$ 12 hours after infection to 5 $\log_{10}$ by day 1 and reached about 6 $\log_{10}$ PFU on day 2. VV-HA-TK titres increased very

rapidly, reaching a mean peak titre of 9 $\log_{10}$ on day 5. About 8 $\log_{10}$ PFU of VV-HA-TK remained in ovaries from day 5 onwards till day 13. In contrast, VV-HA-IL2 titres dropped sharply after the second day of infection with only 3 $\log_{10}$ PFU remaining on day 5 when the VV-HA-TK titre was 9 $\log_{10}$. The IL-2-encoding recombinant virus was not detected in ovaries of nude mice after the sixth day of infection.

Both the recombinant viruses seeded lungs with comparable mean viral titres 0.5, 1 and 2 days after infection (Fig. 3), suggesting that the rates of replication *in vivo* were also comparable during the first 2 days of infection. After 2 days, marked differences were apparent in viral titres. VV-HA-TK increased from 4.8 $\log_{10}$ PFU on day 2 to about 6.5 $\log_{10}$ PFU on day 3 and persisted thereafter at about 6 $\log_{10}$ PFU until day 13 when mice started dying. On the other hand, VV-HA-IL2 titres did not increase beyond the 3rd day of infection, when mean lung titre was about 5 $\log_{10}$ PFU. Viral titres decreased rapidly thereafter and could not be detected in lungs after the 6th day of infection.

The replication of these recombinant viruses in spleens and brains of nude mice showed somewhat a different pattern. VV-HA-IL2 was detected in spleens 12 hours after infection but could not otherwise be recovered from spleens or brains (Fig. 3). VV-HA-TK persisted in the spleens with mean titres between 3-4 $\log_{10}$ PFU except for the 4th day of infection when mean titre was a little over 5 $\log_{10}$ PFU. VV-HA-TK was recovered from brains only after the 4th day of infection, reaching a peak of 6 $\log_{10}$ PFU on day 10 and persisting at close to this level on the 13th day after infection (Fig. 3).

The delayed appearance of VV-HA-TK (5 days after infection) in the brain, together with its rate of apparent replication, suggested that the virus was being seeded from other organs, such as the ovaries and lungs when peak titres were demonstrable (Fig. 3). The absence of VV-HA-IL2 in mouse brains after infection may be attributed not only to its intrinsic inability to maintain replication in the brain, but also to the more efficient clearance of this virus in comparison with VV-HA-TK.

Kinetics of viral replication in normal CBA/H mice.

Female CBA/H mice between 6-8 weeks of age were infected i.v. with 10^7 PFU of VV-HA-TK or VV-HA-IL2. Groups of 4 mice were sacrificed on

the days indicated (Fig. 4), their ovaries, lungs, spleens, and livers removed and virus titres in these organs determined.

In all organs, mean VV-HA-TK and VV-HA-IL2 titres were comparable 1 day after infection.

Compared to the other three organs, VV replicated most efficiently in murine ovaries (Fig. 4). VV-HA-TK replicated rapidly with titres increasing from 4.8 $\log_{10}$ on day 1 to reach a maximum of about 8.6 $\log_{10}$ on day 3. Although clearance of VV-HA-TK from ovaries was apparent by the 4th day of infection, the control virus was detectable at day 10 post-infection. VV-HA-IL2 reached only 5.3 $\log_{10}$ on day 2 and thereafter was cleared more rapidly from ovaries than VV-HA-TK; no virus was recovered after the 6th day of infection.

In the lungs, VV-HA-TK reached a peak titre of 4.9 $\log_{10}$ PFU on day 3 and declined thereafter to undetectable levels on day 6 after infection. Mean peak VV-HA-IL2 titre in the lungs (4 $\log_{10}$) was recorded on the 2nd day, and virus was cleared by the 5th day post-infection.

Although neither virus replicated in spleens or livers as much as in the ovaries, VV-HA-IL2 was cleared from both these organs earlier than VV-HA-TK (Fig. 4).

Infection with VV-HA-IL2 does not result in systemic circulation of IL-2.

In order to study the immune mechanism(s) induced by IL-2 which contributed to nude mice survival and the rapid clearance of VV-HA-IL2 in nude and normal mice, it was of interest to establish whether this lymphokine was present in the serum of infected mice

Sera collected from nude mice given 10^7 PFU of VV-HA-IL2 or VV-HA-TK i.v. 12 hours after infection and thereafter everyday until day 13 contained no detectable IL-2 as assesed by ^{3}H-thymidine incorporation into the IL-2 dependent T-cell line CTL-L (data not shown). Similarly, no murine IL-2 was detected in sera of normal CBA/H mice between 1-10 days after i.v. infection with 10^7 PFU VV-HA-TK or VV-HA-IL2.

The effect of anti-IL-2 Mab treatment on viral titres and survival of nude mice

Data presented above demonstrated that IL-2 was not detected in sera of mice infected with VV-HA-IL2. It was therefore important to establish that IL-2 was indeed produced *in vivo* during an infection with VV-HA-IL2.

Groups of 11 outbred nude mice infected i.v. with 10^7 PFU of VV-HA-IL2 or VV-HA-TK were simultaneously treated with Mab to murine IL-2. The rationale for this approach is as follows. Firstly, treatment with the Mab to IL-2 should reduce the activity of IL-2 produced *in vivo* during infection with VV-HA-IL2. Secondly, if the highly attenuated phenotype of VV-HA-IL2 is due to the influence of and/or mechanism(s) induced by virus-encoded IL-2, then treatment with the antibody should increase the severity of infection.

Results presented in Table 2 indicate that this is indeed the case. All 7 mice infected with VV-HA-IL2 and treated with the Mab to IL-2 died, whereas mice given virus alone survived. Antibody-treated nude mice infected with VV-HA-TK all died with a MTD of 7.3 days which was not significantly different ($p > 0.05$) from those given VV-HA-TK only.

Furthermore, five days after infection, mean titres of VV-HA-IL2 in ovaries and lungs of mice treated with the Mab were significantly higher ($p < 0.001$) than untreated controls (Table 3). Marginal increases were apparent in VV-HA-TK titres in ovaries and lungs of mice treated with the antibody, but were not significantly higher than controls.

These results are consistent with an important role for IL-2-induced immune mechanism(s) in rapid viral clearance and survival of nude mice.

DISCUSSION

The MTD in inbred CBA or outbred Swiss nude mice infected with VV-HA-TK was dose-dependent. Outbred nude mice given $5x10^5$ PFU of VV-HA-TK exhibited a delay in the onset of signs of disease like weight loss and pox lesions on the skin compared to mice given higher doses of virus, but 100% mortality was nevertheless observed.

Although all nude mice given varying doses of VV-HA-TK eventually died from disseminated vaccinial disease, VV-HA-TK was less virulent than the wild type virus, VV-WR. Nude mice given VV-WR died twice as fast as mice given similar doses of VV-HA-TK. Deletion or insertional inactivation of the VV-WR TK gene is known to reduce virus virulence (Buller *et al.*, 1985). VV-HA-TK possesses a TK^+ phenotype via expression of the HSV TK gene. However, the HSV TK is not as efficient as the vaccinia TK since VV-HA-TK expresses only about 10% of the enzyme activity of VV-WR raising the possibility that this is the cause of reduced virulence (Coupar *et al.*, 1987). On the other hand, it is also possible that insertion of the HSV TK gene into the F region of the *Hind* III fragment resulted in reduced virulence. Nevertheless, VV-HA-TK caused 100% mortality in nude mice.

Insertion of the murine IL-2 gene into the vaccinia genome resulted in the most marked 'attenuation' observed here as indicated by the absence of mortality in nude mice infected i.v. with $5x10^7$ PFU of VV-HA-IL2. Also, VV-HA-IL2 was cleared more efficiently than VV-HA-TK by T-cell deficient nude mice, thus enabling them to survive the infection. The difference in clearance efficiency was more marked in nude mice than in normal mice.

Two important inferences were made from experiments involving the use anti-IL-2 Mab to inhibit the function of IL-2 in VV-HA-IL2 infected nude mice. This resulted in 100% mortality from an otherwise sublethal dose of virus. Firstly, it was established that IL-2 was indeed produced *in vivo*. Secondly, the highly attenuated phenotype exhibited by VV-HA-IL2 was not because of 'attenuation' *per se* due to insertional inactivation but rather due to the IL-2-induced mechanism(s) of rapid viral clearance in the host system.

The inability to detect IL-2 in sera of mice infected with VV-HA-IL2 suggested that the lymphokine was operating locally at the sites of virus infection.

Investigation of the kinetics of VV-HA-IL2 clearance from infected nude or normal mice showed that the mechanisms involved in IL-2-mediated clearance of virus were induced and operated rapidly within 1-4 days after infection. This suggested strongly that cell-mediated immune mechanism(s) may have been involved. The rapid clearance of VV-HA-IL2 compared to VV-HA-TK also excluded a role for humoral immunity as previously reported (Ramshaw *et al.*, 1987).

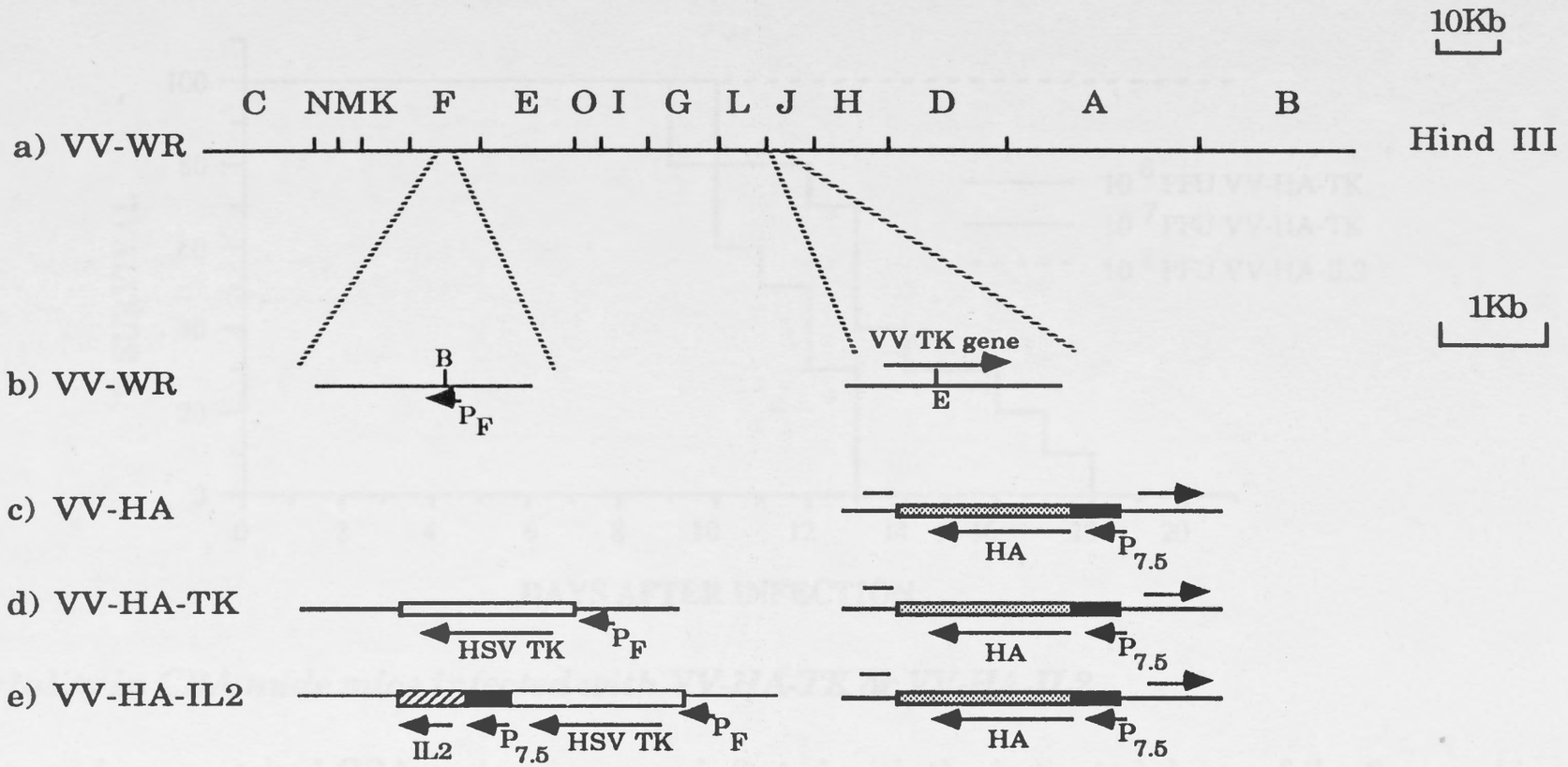


Figure 1. ***Genomic configuration of vaccinia recombinants.***

A *Hind*III map (a) of vaccinia virus, strain VV-WR, is shown with insertion points at the *Eco*RI (E) and *Bam*HI (B) sites in the J and F fragments respectively (b). Arrows indicate orientations of vaccinia virus TK gene, vaccinia promoters P_F and $P_{7.5}$, inserted influenza HA gene, HSV TK and murine IL-2 coding sequences. (Reproduced from Ramshaw *et al.*, 1987)

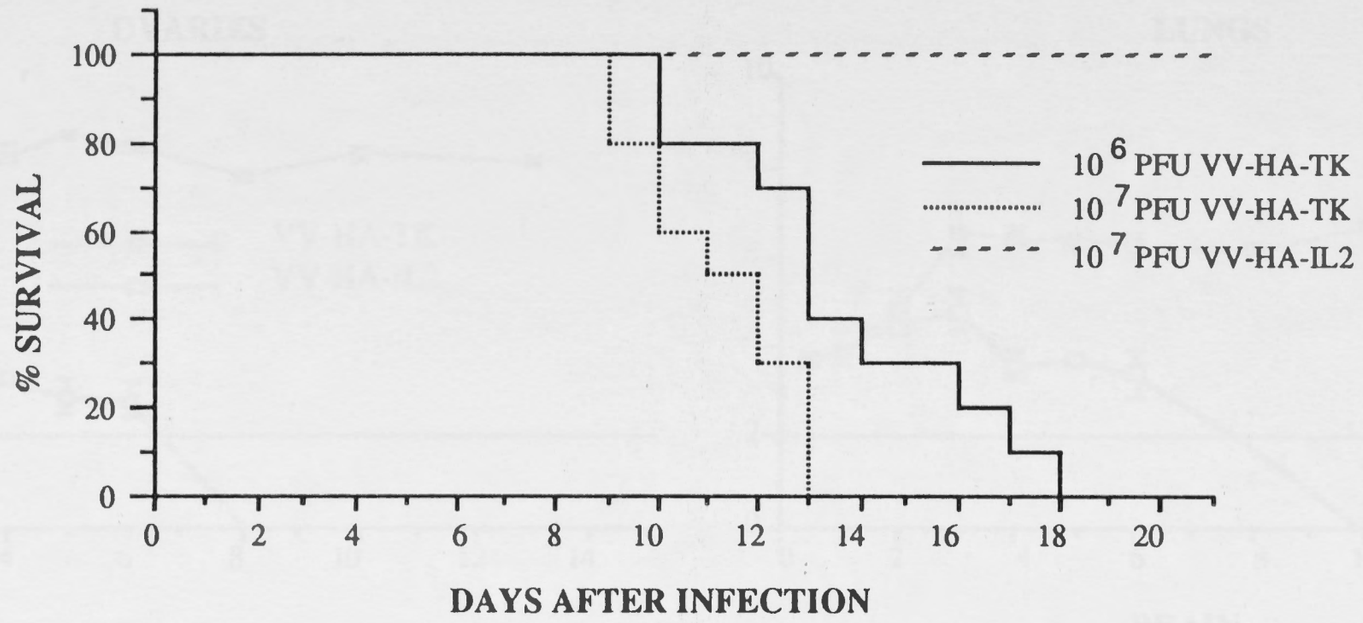


Figure 2. ***Mortality in CBA nude mice infected with VV-HA-TK or VV-HA-IL2.***

Groups of 10 age and sex matched CBA nude mice were infected with the indicated doses of the 2 recombinant viruses and morbidity and mortality were monitored. Nude mice infected with VV-HA-IL2 showed no signs of morbidity and no mortality was recorded beyond the 21 day observation period.

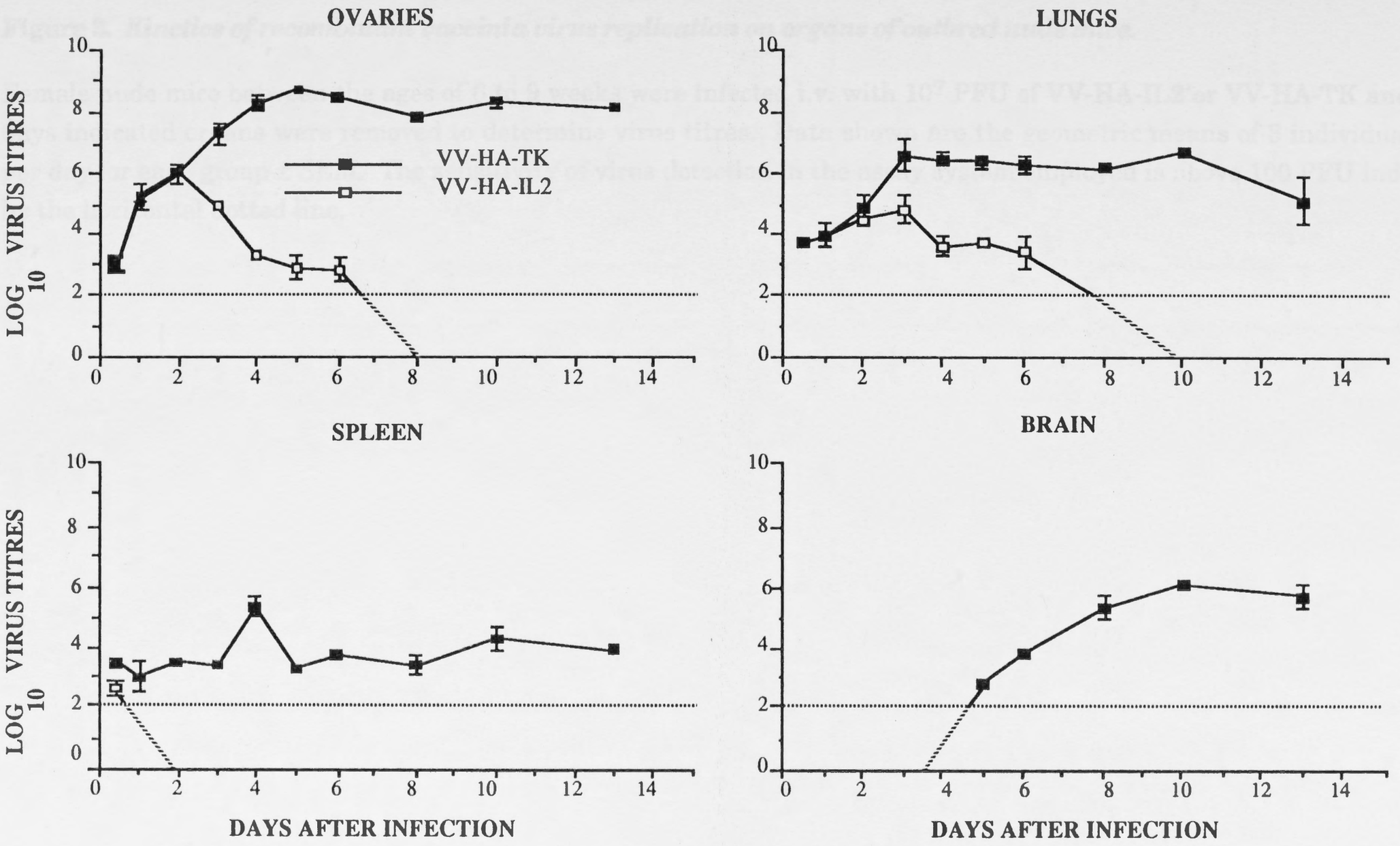
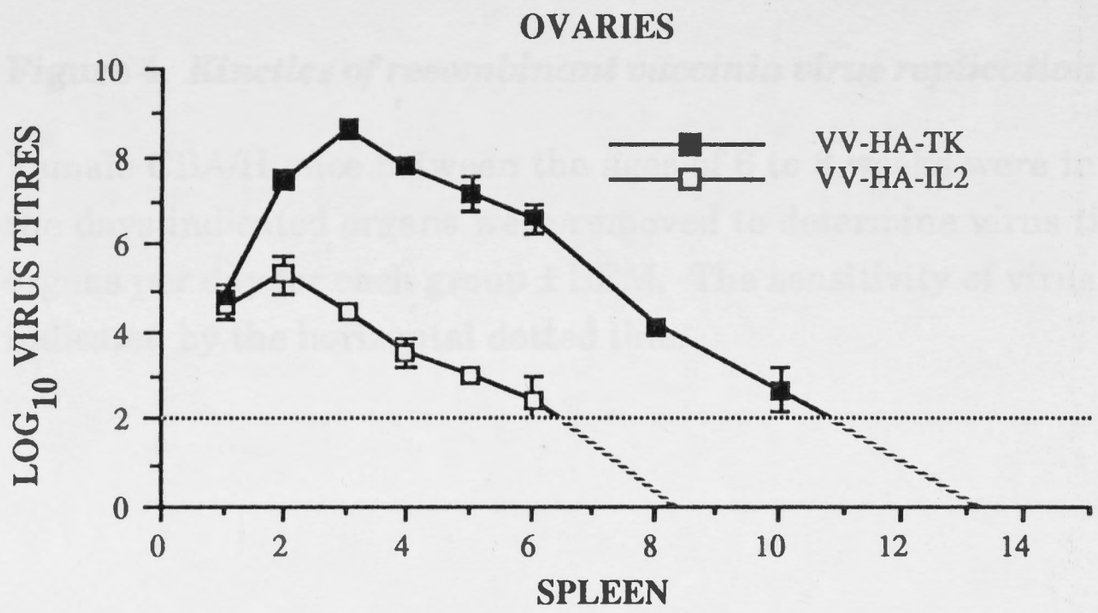

OVARIES
LUNGS
SPLEEN
BRAIN
LOG 10 VIRUS TITRES
VV-HA-TK
VV-HA-IL2
DAYS AFTER INFECTION
DAYS AFTER INFECTION

Figure 3. ***Kinetics of recombinant vaccinia virus replication on organs of outbred nude mice.***

Female nude mice between the ages of 6 to 9 weeks were infected i.v. with 10^7 PFU of VV-HA-IL2 or VV-HA-TK and on the days indicated organs were removed to determine virus titres. Data shown are the geometric means of 3 individual organs per day for each group ± SEM. The sensitivity of virus detection in the assay system employed is above 100 PFU indicated by the horizontal dotted line.


OVARIES
VV-HA-TK
VV-HA-IL2
LOG10 VIRUS TITRES
SPLEEN
DAYS AFTER INFECTION

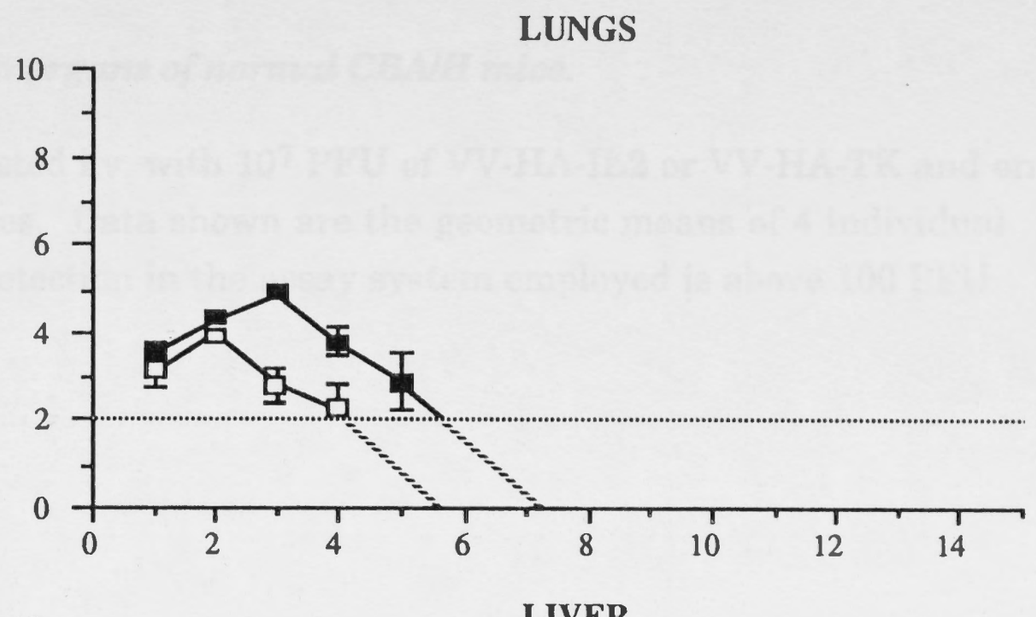

LUNGS
LIVER
DAYS AFTER INFECTION

Figure 4. ***Kinetics of recombinant vaccinia virus replication on organs of normal CBA/H mice.***

Female CBA/H mice between the ages of 6 to 8 weeks were infected i.v. with 10^7 PFU of VV-HA-IL2 or VV-HA-TK and on the days indicated organs were removed to determine virus titres. Data shown are the geometric means of 4 individual organs per day for each group ± SEM. The sensitivity of virus detection in the assay system employed is above 100 PFU indicated by the horizontal dotted line.

Table 1.

Mortality in nude mice given different doses of wild type or recombinant vaccinia viruses.

Virus (Dose)[a]	No. of mice	% Mortality	MTD (death on day p.i.)
VV-WR (10^6 PFU)	7	100	8.9 (8,8,9,9,9,9,10)
VV-WR (10^7 PFU)	7	100	7.4 (6,7,7,7,8,8,9)
VV-HA-TK ($5x10^5$ PFU)	7	100	23.0 (17,18,22,24,26,26,28)
VV-HA-TK (10^6 PFU)	7	100	17.8 (15,16,16,17,19,20,22)
VV-HA-TK ($5x10^6$ PFU)	7	100	15.6 (12,12,13,16,17,19,20)
VV-HA-TK (10^7 PFU)	7	100	14.8 (12,12,12,15,17,17,19)
VV-HA-IL2 (10^7 PFU)	7	0	-
VV-HA-IL2 ($5x10^7$ PFU)	7	0	-

[a] Age and sex matched outbred nude mice were infected i.v. with the indicated doses of virus. No morbidity or mortality was observed in groups that received VV-HA-IL2 beyond the 30 day observation period.

Table 2.

The effect of anti-IL-2 Mab treatment on the survival of nude mice infected with VV-HA-IL2 or VV-HA-TK. [a]

No of mice	Virus	Anti-IL-2 Mab treatment	% Survival	MTD [b] (death on days p.i.)[c]
7	VV-HA-IL2	-	100	-
7	VV-HA-IL2	+	0	6.9 (5,5,6,7,8,8,9)
7	VV-HA-TK	-	0	7.6 (6,7,7,7,8,8,10)
7	VV-HA-TK	+	0	7.3 (6,6,7,7,7,9,9)

[a] Four to 5 week old outbred nude mice were injected i.v. with 10^7 PFU VV-HA-IL2 or VV-HA-TK. On days 0 (day of infection), 1, 2, 3 and 4, mice were given i.p. approximately 500μg of anti-IL-2 Mab (+) and controls were left untreated (-). Mortality was monitored in all groups. Nude mice treated with anti-IL-2 Mab only all survived.

[b] Mean time to death.

[c] Numbers in parentheses indicate the days on which individual mice died.

Table 3.

The effect of anti-IL-2 Mab treatment on VV-HA-IL2 and VV-HA-TK titres in nude mice.

Virus infection and treatment [a]	Log_{10} virus titres ± SEM	
	ovaries	lungs
VV-HA-TK	8.3 ± 0.1	6.3 ± 0.1
VV-HA-TK ± anti-IL-2 Mab	8.8 ± 0.2	6.7 ± 0.3
VV-HA-IL2	3.0 ± 0.1	3.3 ± 0.1
VV-HA-IL2 ± anti-IL-2 Mab	7.8 ± 0.4	5.6 ± 0.2

[a] Four to 5 week old mice were infected with virus and treated with anti-IL-2 Mab as described for Table 2. On day 5 post-infection, mice were sacrificed and virus titres in lungs and ovaries were determined.

[b] Data shown are the geometric means of 4 individual organs per group ± SEM.

[c] Increase in titre highly significant ($p < 0.001$; Students T-test) compared to untreated group infected with VV-HA-IL2 only.

CHAPTER 3

Tc cell and NK cell responses in nude and normal mice infected with recombinant VV

INTRODUCTION.

Cytokines identified to date, including IL-2, have all been shown, at least *in vitro*, to have multiple biological activities. IL-2 is pivotal for the generation and regulation of an immune response (Smith, 1988). It has direct stimulatory effects on T cells (Morgan *et al.*, 1976), B cells (Mingari *et al.*, 1985), monocytes (Holter *et al.*, 1987), organ-derived macrophage precursors (Baccarini *et al.*, 1988), and NK cells (Hefeneider *et al.*, 1983). Because of its pleiotropic effects on various lymphoid and myeloid cell populations, both *in vivo* and *in vitro*, IL-2 may have numerous immunomodulatory effects in immunodeficient or immunocompetent mice infected with VV-HA-IL2. These may be narrowed, however, when considering immune mechanisms induced in response to viral infection as in the present context.

The experiments described in this chapter were designed to elucidate mechanism(s) influenced or induced by IL-2 (during infection with VV-HA-IL2) which may contribute to the process of rapid viral clearance in nude and normal mice. The role of humoral immune mechanisms has been excluded for reasons discussed in the preceding chapter.

Theoretically, the expression of IL-2 during infection of normal mice with VV-HA-IL2 may have resulted in enhanced Tc cell responses or accelerated their generation. Such modified responses could have contributed to the rapid clearance of VV-HA-IL2. Reports that nude mice given allogeneic cells plus IL-2 can develop allospecific Tc cells (Wagner *et al.*, 1980b) suggested that antiviral Tc cells might be induced in VV-HA-IL2-infected nude mice.

Although young adult, athymic, nude mice lack functional T cells, they are detectable in aged nude mice by 4-6 months of age (Gillis *et al.*, 1979; Hunig and Bevan, 1980; Maryanski *et al.*, 1981; MacDonald and Lees, 1984; Chen *et al.*, 1984). The expression of alloreactivity by nude T cells is well established (Hunig and Bevan, 1980; Hale, 1980; Maryanski *et al.*, 1980) though the precursor frequency of alloreactive Tc cells in nude mice is very low compared to that seen in normal mice. The existence of functional T cells in athymic mice has been taken as evidence that some T lymphocytes can develop via an extra-thymic pathway.

Another possibility is that activation of NK cells by IL-2 could contribute to antiviral immunity (Weinberg *et al.*, 1986; Bukowski *et al.* 1988). This applies to both nude and normal mice infected with VV-HA-IL2.

More recently, there have been reports that administration of IL-2 in mice or the exposure of lymphoid cells to IL-2 *in vitro*, results in the generation of LAK cells (Rosenberg *et al.*, 1985; Merluzzi *et al.*, 1984; Mazumder and Rosenberg, 1984; Thompson *et al.*, 1986). Adoptive transfer of cultured LAK cells to MCMV-infected mice substantially reduced virus titres (Bukowski *et al.*, 1988). While precursors of LAK cells from nude mice appear to be NK cells (Hasui *et al.* 1989), those from normal mice are mainly NK cells and some T cells (Kalland *et al.*, 1987; Lotzova and Heberman, 1987).

The induction of Tc and NK cell responses in nude and normal mice infected with VV-HA-TK or VV-HA-IL2 was investigated using a kinetic approach. By using a panel of LAK cell sensitive (NK cell insensitive) targets, the induction of LAK cell activity was assessed. Finally, the phenotypes of the various cytolytic populations induced by VV in nude and normal mice were analysed.

MATERIALS AND METHODS

Mice.

As described in Chapter 2.

Viruses.

As described in Chapter 2.

Cell lines.

L929 (H-2^k), a continuous fibroblast line from C3H mouse (Sanford *et al.*, 1948), YAC-1 (H-2^a), a line derived from Moloney leukemia virus-induced lymphoma in A/Sn mouse (Kiessling *et al.*, 1975), P815 (H-2^d), a mastocytoma line derived from DBA/2 mouse (Potter and Dunn, 1957), and EL-4 (H-2^b), a lymphoma line derived from C57BL/6N mouse (Gorer, 1950), were all maintained in Eagle's minimum essential medium (EMEM) (Gibco, Grand Island, NY, USA) supplemented with antibiotics and 5% heat-inactivated FCS (Flow Laboratories, North Ryde, Australia) and used as targets in cytotoxicity assays.

Antibodies.

The following Mabs and antiserum were used at the dilutions indicated for *in vivo* cell depletion with complement: anti-Thy-1.2, clone F7D5 (Serotec Ltd., Blackthorn Bicester, England) at a dilution of 1:1000; anti-CD4, clone RL174 (Ceredig *et al.*, 1985) and anti-CD8, clone 31M (Sarmiento *et al.*, 1980) kindly provided by Dr. R. Ceredig (John Curtin School of Medical Research) at dilutions of 1:10; and rabbit anti-ganglio-tetraosylceramide (anti-asialo-GM_1 or anti-as-GM_1) (Wako Pure Chemicals Industries, Osaka, Japan) at a dilution of 1:50 (1:25 or 1:10 for some experiments). Low toxicity rabbit complement (C) (Cedarlane Laboratories, Ltd., Canada) was used at a final dilution of 1:10.

Preparation of effector cells from spleens for cytotoxicity assays.

Single cell suspensions from pooled spleens were obtained by gently pressing minced spleens through stainless steel mesh with syringe plungers. Erythrocytes and dead cells were removed by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient separation and the remaining cells at the inter-phase were removed, washed 3 times with

EMEM supplemented with 5% heat-inactivated FCS, 10 mM HEPES buffer and antibiotics (complete medium). After the final wash, cells were resuspended in complete medium at the desired concentrations.

Depletion of effector cell suspensions with antibody and complement.

Effector cells suspended in 1 ml of EMEM at the appropriate concentrations, were treated with antibodies for 30 min at 4°C, washed once and resuspended in 1 ml of EMEM containing rabbit C. After incubation for 45 min at 37°C, the cells were washed twice and resuspended in 2 ml of EMEM without further adjusting the concentrations.

Cytotoxicity assays.

Target cells were labelled with ^{51}Cr (about 50μCi for $2x10^6$ cells) for 1 h at 37°C in a humidified atmosphere containing 5% CO_2 in air, washed 3 times in complete medium and resuspended at a concentration of $2x10^5$ cells/ml for use as targets. For virus-infected targets, L929 cells were infected with VV-WR at 20-40 PFU/cell during the 1 h labelling period. The standard chromium release assay was carried out in triplicate in U-bottom microtitration plates ($2x10^4$ target cells plus the appropriate number of splenocyte effector cells) for 4 h with YAC-1 targets and 6 hours for L929 (infected or uninfected), EL-4 and P815 targets, at 37°C in a humidified atmosphere of 5% CO_2 in air. ^{51}Cr release from targets was measured in a Packard Auto-Gamma counter and specific lysis was calculated using the formula

$$\% \text{ specific lysis} = \frac{\text{experimental release - spontaneous release}}{\text{maximal release - spontaneous release}} \times 100$$

Spontaneous release ranged from 6-10% for YAC-1 cells, 8-12% for L929 cells, and 7-15% for P815 and EL-4 cells.

RESULTS

Tc cell responses in nude mice.

To investigate the possibility that IL-2 expressed during infection with VV-HA-IL2 induced anti-viral Tc cell responses in nude mice, inbred CBA nude mice were infected i.v. with 10^6 PFU of VV-HA-TK or VV-HA-IL2 and the kinetics of Tc cell generation and cytolytic activity were monitored. Splenocyte populations were obtained from groups of 4 mice infected with VV-HA-TK or VV-HA-IL2 1, 2, 3, 4, 5, 6 and 8 days previously and their ability to lyse uninfected or VV-infected H-2-compatible (L929) targets was assessed.

Splenocyte effector cells obtained from nude mice 1-8 days after infection with VV-HA-IL2 lysed uninfected and VV-infected L929 targets and the level of cytolysis was consistently 3-fold higher than that seen with VV-HA-TK-induced effectors (Table 1). The lysis of uninfected or infected targets by VV-HA-TK-immune effectors was higher than lysis by effectors obtained from unimmunized control mice. Although VV-infected targets were lysed slightly more efficiently by splenocytes from VV-HA-TK or VV-HA-IL2 infected mice, the levels of cytolysis of uninfected or infected targets were not significantly different. The peak cytolytic activity was observed at 5-6 days after infection. The finding that uninfected targets were lysed by the effector cell populations indicated that lysis was not caused by virus-specific Tc cells.

Phenotypic analysis revealed that $CD4^+$ or $CD8^+$ T cells in the splenocyte population were not responsible for the lysis of VV-infected or uninfected targets (Table 2), since treatment with anti-CD4 or anti-CD8 monoclonal antibody and C did not abrogate the cytolytic activity. However, treatment with anti-Thy-1.2 or anti-as-GM_1 and C resulted in a 3-fold reduction of the cytolytic activity of 6-day immune, VV-HA-IL2-induced effectors. On the contrary, while most of the cytolytic activity of 6-day VV-HA-TK-immune effectors was eliminated after treatment with anti-as-GM_1 and C, these effectors were more resistant to treatment with anti-Thy-1.2 and C. Splenocytes obtained from nude mice 2 days after infection with VV-HA-TK or VV-HA-IL2 bore similar phenotypes to 6 day-immune splenocyte effectors that mediated lysis of uninfected or infected L929 target cells (data not shown). Thus, the IL-2 expressed during infection

with VV-HA-IL2 did not induce the generation of classical antiviral $CD8^+$ Tc cells in nude mice.

Tc cell responses in normal CBA/H mice.

In the preceding chapter, it was established that in normal mice, VV-HA-IL2 was cleared a few days earlier than VV-HA-TK. Thus, the possibility that IL-2 secreted *in vivo* during infection with VV-HA-IL2 accelerated the generation of Tc cells or perhaps enhanced Tc cell responses in normal mice was investigated.

The kinetics of Tc cell generation and activity in normal CBA/H mice given VV-HA-TK or VV-HA-IL2 were examined. Splenocytes were obtained from groups of 4 mice infected i.p. with 10^7 PFU of VV-HA-TK or VV-HA-IL2, 1, 2, 3, 4, 5, 6 and 8 days earlier, and the ability of effectors to lyse uninfected or VV-infected L929 targets was determined.

Both VV-HA-IL2- and VV-HA-TK-induced effectors lysed uninfected as well as infected L929 targets from as early as 1 day after infection (Table 3). The lysis of uninfected L929 targets by VV-HA-IL2-immune splenocytes was about 3-fold higher than lysis caused by VV-HA-TK-immune spleen cells, peaked at days 2-3 after infection, and declined to control (unimmunized) levels after the 5^{th} day. Lysis of virus-infected targets over and above uninfected targets by VV-HA-IL2-immune cytotoxic Tc was demonstrable on the 4^{th} day after infection, peaked at days 5-6 and persisted on day 8 after infection. However, some of the cytolytic activity on VV-infected targets on days 4 and 5 may be attributed to non-specific cytolysis, as illustrated by lysis of uninfected targets on the same days.

The cytolytic activity of splenocytes from mice given VV-HA-TK on uninfected L929 targets was about 3-fold higher than the activity exhibited by unimmunized control splenocytes on the first and second days after infection. Cytolytic activity dropped to below controls levels by the 5^{th} day of infection (Table 3). Antiviral Tc cell responses in normal mice given VV-HA-TK appeared on the 3^{rd} day after infection, peaked at day 6 and were still strong on the 8^{th} day. That lysis of virus-infected targets by 2-day-immune splenocytes from normal mice infected with VV-HA-TK or VV-HA-IL2 was not due to classical $CD8^+$ Tc cells was confirmed by phenotypic analysis. Treatment with anti-CD8 antibody and C did not

affect the cytolytic activity. Six-day immune splenocytes (VV-HA-TK or VV-HA-IL2), however, were sensitive to both anti-CD8 and Thy-1.2 and C (Table 5) thus confirming that the lysis of H-2-compatible, virus-infected targets was mediated by classical Tc cells. The above data established that IL-2 produced during infection with VV-HA-IL2 in normal mice did not accelerate the generation of Tc cells or enhance their response in comparison to infection with VV-HA-TK.

NK cell responses.

The lysis of uninfected L929 target cells by splenocytes from mice immunized with VV-HA-IL2 was consistently about 3-fold higher than lysis due to VV-HA-TK-induced effectors on days 1, 2 and 3 . This was true for nude mice (Table 1), with greater cytolytic activity observed over a period of 8 days after infection, and normal mice (Table 3). Since NK cells are known to be part of the early response to infection (reviewed in Welsh., 1981), further characterisation of NK responses was undertaken.

Splenocytes from CBA nude mice infected i.v. with 10^6 PFU of VV-HA-IL2 exhibited elevated (3-fold) cytolytic activity on YAC-1 target (classical NK targets) cells compared to splenocytes from VV-HA-TK-infected mice or uninfected controls (Table 6). This elevated cytolytic activity persisted for at least 6 days after infection.

In normal mice infected i.p. with 10^7 PFU of VV-HA-IL2, NK cell activity (lysis of YAC-1 targets) was also about 3-fold higher for the first 3 days than in mice given VV-HA-TK (Table 6). Cytolytic activity decreased to below levels of unimmunized controls by day 6 after infection when Tc activity peaked (Table 3). In addition, the cytolytic activity on YAC-1 targets mediated by VV-HA-IL2- and VV-HA-TK-immune effectors 1, 2, 3 and 6 days after infection correlated with the lysis of uninfected L929 targets on these days indicated (Table 3).

Phenotype of effectors from nude or normal CBA mice mediating lysis of YAC-1 targets.

Using the panel of anti-serum and Mab and C, the general finding was that effectors mediating lysis of YAC-1 targets were mainly Thy-1.2$^{\pm}$, CD4^{-}, CD8^{-} and as-GM$_1^{+}$ (Table 7), the phenotype of conventional NK cells. However, effectors from VV-HA-IL2-infected mice were more sensitive to

anti-Thy-1.2 and C and less sensitive to anti-as-GM_1 and C than cells from mice infected with VV-HA-TK or from uninfected controls. In the case of as-GM_1, this phenomenon may have reflected quantitative differences in expression of the ligand on the cell surface, because more effectors could be lysed by increasing the antiserum concentration, resulting in reduced levels of YAC-1 target lysis (Table 8). However, some cytolytic activity still remained despite the use of higher concentrations of antiserum.

NK cell responses in outbred nude mice.

The kinetics of NK cell generation and cytolytic activity in outbred nude mice (Table 9) were similar to those seen in inbred CBA nude mice (Tables 1 and 6). Lysis of YAC-1 targets by VV-HA-IL2-immune splenocytes was at least 3-fold higher than that mediated by VV-HA-TK-immune cells and the elevated activity persisted, being detectable even on day 8 after infection. Peak cytolytic activity was observed 5 days after infection with VV-HA-IL2 and 3 days after VV-HA-TK. Furthermore, phenotypic analysis of 3- and 5-day immune spleen cells indicated that the effectors which lysed YAC-1 targets were mainly Thy-1.2$^{\pm}$, as-GM_1^+, CD4$^-$ and CD8$^-$ (Table 10). Increasing the concentration of the anti-as-GM_1 to remove as-GM_1^+ cells further reduced cytolytic activity on YAC-1 targets (Table 8).

Lysis of P815, EL-4 and L929 targets by VV-HA-IL2-induced splenic effector cells from outbred nude mice.

The ability of VV-HA-IL2-induced effectors to lyse targets such as P815 and EL4 was tested. These cells are usually regarded as insensitive to NK cells. Results obtained using normal mice infected with VV-HA-IL2 or VV-HA-TK were variable between experiments, but generally spleen cells obtained during the first 3 days after infection caused low but insignificant levels of lysis of P815 and EL-4 targets (data not shown). However, splenocytes obtained from outbred nude mice given 10^7 PFU of VV-HA-IL2 i.v. caused significant lysis of P815, EL4 and L929 targets, though at lower levels than lysis of YAC-1 targets (Fig. 1). Effectors obtained from VV-HA-TK-infected nude mice showed significant cytolytic activity only on YAC-1 targets. The VV-HA-IL2-induced cytolytic splenocytes mediating lysis of P815, EL-4 and L929 targets had the same

phenotype (data not shown) as effectors which lysed YAC-1 targets, i.e., as-GM_1^+, Thy-$1.2^{\pm}$, $CD4^-$ and $CD8^-$.

DISCUSSION.

Young adult, athymic, nude mice lack functional T-cells which are normally essential for recovery from primary infection with pox viruses (Hirsh *et al.*, 1968, Blanden, 1970; 1971a, b). Therefore, infection with VV results in persistence of virus, disseminated vaccinial disease, and death (Ramshaw *et al.*, 1987; and Chapter 2 , Table 1). However, when infected with recombinant VV encoding murine IL-2, nude mice resolve the infection (Ramshaw *et al.*, 1987; and Chapter 2, Table 1). Evidence presented in this chapter established that nude mice recover from infection with VV-HA-IL2 without detectable Tc cell responses.

Inbred nude mice infected with VV-HA-IL2 or VV-HA-TK had no detectable antiviral Tc cell responses during the first eight days after infection. Nude mice given allogeneic cells plus IL-2 were reported to mount an alloreactive Tc cell response (Wagner *et al.*, 1980b). Here, IL-2 was used primarily to expand the small pool of alloreactive T cell precursors. Effectors which lysed appropriate targets *in vitro* were Thy-1.2^+ but no attempt was made to demonstrate the presence of CD8 (Ly2) antigen. In their studies, Wagner *et al.* did not include control mice that were given only IL-2 although a group given only allogeneic cells failed to respond. In the light of current knowledge of the effects of IL-2 on nude mouse lymphoid cells, i.e. induction of cytolytic cells which lyse a broad range of targets in a non-MHC-restricted fashion (Hasui *et al.*, 1989), and the fact that some NK cells also express the Thy-1.2 antigen (Hackett *et al.*, 1986), their data will have to be reevaluated. Infection with VV-HA-IL2 failed to induce detectable anti-VV Tc cell responses in nude mice. Virus-encoded IL-2 should theoretically expand a limited pool of precursors, if indeed there were any, but they remained undetectable in the present experiments.

Normal mice infected with VV-HA-IL2 or VV-HA-TK generated strong anti-VV Tc cell responses, with peak cytolytic activity on days 5-6 as reported previously for wild type virus, VV-WR (Koszinowski and Thomssen, 1975). However, infection with VV-HA-IL2 did not enhance

Tc cell responses or accelerate their generation in comparison with VV-HA-TK. This finding is consistent with earlier reports that the expression of IL-2 by recombinant VV enhanced neither the Tc cell responses in normal mice (Müllbacher *et al.*, 1989) nor the proliferative responses of Th cells in chimpanzees (van Eendenburg *et al.*, 1989). In both reports, however, the rate of clearance of control and IL-2-encoding recombinant viruses was not addressed. Although VV-HA-IL2 is cleared more efficiently than VV-HA-TK in normal mice, anti-vaccinia Tc cell responses were comparable at 5-6 days after infection.

The enhanced NK activity observed in spleens of mice given VV-HA-IL2 compared to VV-HA-TK can be attributed to the well-documented augmentation of NK activity by IL-2 *in vivo* (Hefeneider *et al.*, 1983; Biron *et al.*, 1990) and *in vitro* (Henny *et al.*, 1981).

In nude mice, the elevated NK activity in response to infection with VV-HA-IL2 persisted for at least 8 days after infection. This could be attributed to the longer persistence of virus in nude mice than in normal mice. Furthermore, normal mice also mount an antiviral Tc cell response which seems to down-regulate the NK cell response. During systemic viral infections, NK cell responses are markedly augmented and this has been generally attributed to IFN-α or -β-mediated activation (MacFarlan *et al.*, 1977; Gidlund *et al.*, 1978). From the data presented here, it appears that in normal mice, Tc cell activity appears when NK cell activity is on the decline and peak Tc cell activity is evident when NK activity is barely detectable.

With respect to the killing of the classical NK-susceptible target, YAC-1, the phenotype of splenocyte effectors from infected nude or normal mice was as-GM_1^+, Thy-1.2$^{\pm}$, CD4$^-$ and CD8$^-$. VV-HA-IL2 induced these cells more efficiently than VV-HA-TK and increased their sensitivity to treatment with anti-Thy-1.2 plus C. The increase in sensitivity could have been due to an expansion of the Thy-1.2$^+$ cell population or induction or upregulation of Thy-1.2 expression. This is consistent with earlier observations concerning the induction of Thy-1.2 expression on splenocytes of nude mice by IL-2 (Wagner *et al.*, 1980b, Lipsick and Nathan, 1981). However, it must be emphasized that there was no evidence that these Thy-1.2$^+$ cells from nude mice were classical antiviral Tc cells as they were CD8$^-$. In the case of nude or normal mice of the CBA

(H-2^k) strain infected with VV-HA-IL2, there was lysis of H-2^k-compatible L929 target cells whether or not they were infected with VV-WR.

In addition, other target cells, such as P815 and EL-4, were lysed by VV-HA-IL2-induced effector cells obtained from nude mice. These two target cell types are generally insensitive to lysis by classical NK cells and cytolytic cells which lyse them have been generally designated LAK cells. Lysis of P815, EL-4 and L929 targets by VV-HA-IL2-induced killers was, however, at far lower levels than lysis of YAC-1 targets, and this, incidentally, has been documented to be typical of activated NK cells (Bukowski *et al.*, 1988). Since the VV-HA-IL2-induced effectors mediating lysis of P815, EL-4 and L929 targets had the same phenotype as effectors which lysed YAC-1 targets, they will therefore be referred to as NK, NK-like or activated NK cells.

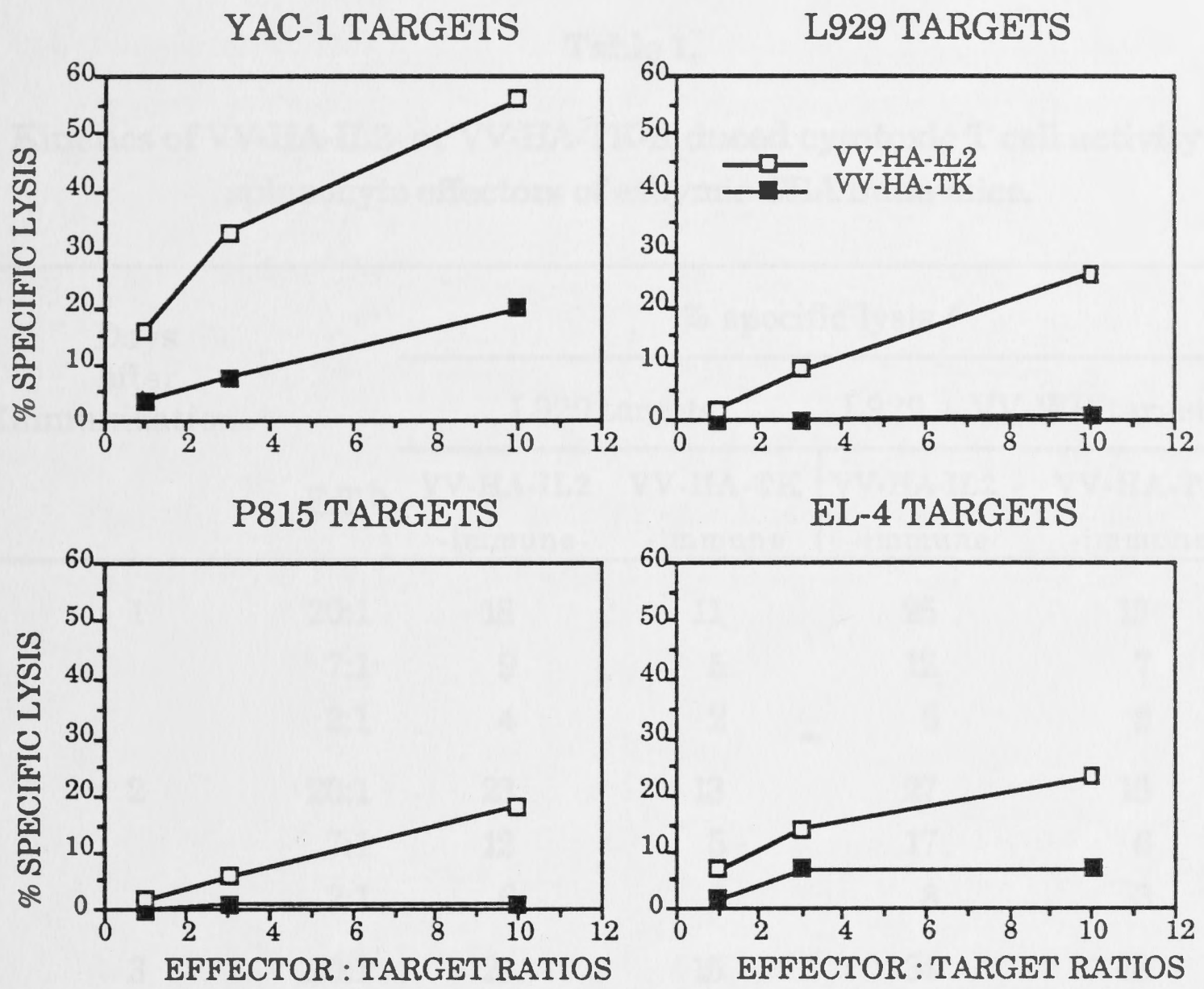


Figure 1. ***Lysis of NK sensitive (YAC-1) and NK insensitive (L929, P815, EL-4) targets.***

Six-week old outbred nude mice were injected i.v. with 10^7 PFU of VV-HA-TK or VV-HA-IL2 and 5 days later, the ability of splenocytes (pooled from 4 spleens) to lyse the various targets was tested. VV-HA-IL2-immune effectors lysed all 4 targets at significant levels whereas VV-HA-TK-induced effectors only lysed YAC-1 targets at significant levels. Data shown are means of triplicates and SEM were less than 5%.

Table 1.

Kinetics of VV-HA-IL2- or VV-HA-TK-induced cytotoxic T cell activity in splenocyte effectors of athymic CBA nude mice.

Days after Immunization [a]	E:T [b]	% specific lysis [c]			
		L929 targets		L929 + VV-WR targets	
		VV-HA-IL2 -immune	VV-HA-TK -immune	VV-HA-IL2 - immune	VV-HA-TK -immune
1	20:1	18	11	25	13
	7:1	9	5	12	7
	2:1	4	2	6	3
2	20:1	23	13	27	16
	7:1	12	5	17	6
	2:1	6	3	8	3
3	20:1	29	15	34	18
	7:1	17	7	19	10
	2:1	9	4	10	5
4	20:1	35	23	41	25
	7:1	21	9	26	12
	2:1	12	6	15	8
5	20:1	54	20	58	23
	7:1	30	12	38	15
	2:1	18	8	22	9
6	20:1	52	25	59	27
	7:1	29	7	33	9
	2:1	20	3	24	6
8	20:1	48	22	51	24
	7:1	26	6	29	7
	2:1	13	4	15	3
Unimmunized control	20:1	8		11	
	7:1	4		5	
	2:1	1		2	

Table 1, cont.

[a] Eight to 10 week old female nude mice were given i.v. 10^6 PFU of VV-HA-IL2 or VV-HA-TK.

[b] Effector : target ratio.

[c] SEM were less than 5% and have been omitted for clarity.

Table 2.

Phenotype of 5-day immune splenocyte effectors from nude mice infected with VV-HA-IL2 or VV-HA-TK.

Treatment [a]	E:T [b]	% specific lysis [c]			
		L929 targets		L929 + VV-WR targets	
		VV-HA-IL2 -immune	VV-HA-TK -immune	VV-HA-IL2 - immune	VV-HA-TK -immune
C	20:1	44	16	49	19
	7:1	27	9	31	11
	2:1	13	3	15	4
C + anti-Thy1.2	20:1	29	12	33	13
	7:1	15	7	15	8
	2:1	8	1	9	2
C + anti-CD4	20:1	49	18	51	20
	7:1	28	10	29	12
	2:1	15	3	15	5
C + anti-CD8	20:1	46	15	48	18
	7:1	25	8	27	9
	2:1	12	4	13	5
C + anti-as-GM_1	20:1	25	3	29	3
	7:1	10	1	13	2
	2:1	4	0	6	1

[a] Six-day immune splenocytes were treated with antibody + C as described in Materials and Methods.

[b] Effector : target ratio.

[c] SEM were less than 5%.

Table 3.

Kinetics of VV-HA-IL2- or VV-HA-TK-induced cytotoxic T cell activity in splenocyte effectors of euthymic CBA/H mice.

Days after Immunization [a]	E:T [b]	% specific lysis [c]			
		L929 targets		L929 + VV-WR targets	
		VV-HA-IL2 -immune	VV-HA-TK -immune	VV-HA-IL2 - immune	VV-HA-TK -immune
1	20:1	22	14	26	18
	7:1	16	7	18	11
	2:1	6	4	10	6
2	20:1	35	12	38	19
	7:1	15	7	19	10
	2:1	9	5	8	5
3	20:1	32	8	36	28
	7:1	15	6	22	10
	2:1	10	4	9	7
4	20:1	27	7	49	42
	7:1	13	5	33	22
	2:1	8	3	15	13
5	20:1	17	3	67	66
	7:1	8	3	49	41
	2:1	6	1	28	24
6	20:1	6	3	64	70
	7:1	3	2	46	49
	2:1	4	2	24	29
8	20:1	4	3	55	64
	7:1	2	3	35	46
	2:1	0	0	18	23
Uninfected control	20:1	8		12	
	7:1	5		7	
	2:1	3		2	

Table 3 cont.

[a] Mice were infected i.p. with 10^7 PFU of virus.

[b] Effector : target ratio.

[c] SEM were less than 5%.

Table 4.

Phenotype of 2-day immune cytotoxic spleen cells from normal mice infected with VV-HA-IL2 or VV-HA-TK.

Treatment [a]	E:T [b]	% specific lysis [c]			
		Uninfected		VV-WR Infected	
		VV-HA-IL2 -immune	VV-HA-TK -immune	VV-HA-IL2 - immune	VV-HA-TK -immune
C	20:1	41	20	47	26
	7:1	22	12	27	16
	2:1	10	4	13	7
C + anti-Thy1.2	20:1	15	12	18	14
	7:1	7	7	9	10
	2:1	3	2	4	4
C + anti-CD4	20:1	38	22	40	25
	7:1	23	10	26	13
	2:1	9	3	12	6
C + anti-CD8	20:1	40	19	43	20
	7:1	24	11	29	14
	2:1	10	4	12	7
C + anti-as-GM_1	20:1	19	2	22	3
	7:1	9	0	13	1
	2:1	5	0	7	0

[a] Six-day immune splenocytes were treated with antibody + C as described in Materials and Methods.

[b] Effector : target ratio.

[c] SEM were less than 5%.

Table 5.

Phenotype of 6-day immune cytotoxic spleen cells from normal mice infected with VV-HA-IL2 or VV-HA-TK.

Treatment [a]	E:T [b]	% specific lysis [c]			
		L929 targets		VV-WR-infected L929 targets	
		VV-HA-IL2 -immune	VV-HA-TK -immune	VV-HA-IL2 - immune	VV-HA-TK -immune
C	20:1	8	11	68	75
	7:1	4	5	48	53
	2:1	1	3	24	29
C + anti-Thy1.2	20:1	3	6	12	15
	7:1	2	3	6	7
	2:1	0	2	2	5
C + anti-CD4	20:1	9	12	63	79
	7:1	4	5	46	51
	2:1	2	3	25	30
C + anti-CD8	20:1	8	11	10	12
	7:1	5	6	4	7
	2:1	1	4	2	4
C + anti-as-GM_1	20:1	6	9	63	70
	7:1	3	4	45	48
	2:1	0	2	20	25

[a] Six-day immune splenocytes were treated with antibody + C as described in Materials and Methods.

[b] Effector : target ratio.

[c] SEM were less than 5%.

Table 6.

Lysis of YAC-1 targets by spleen cells from mice immunized with VV-HA-IL2 or VV-HA-TK.

Days after Immunization [a]	E:T [b]	% specific lysis [c]			
		Effectors from Euthymic CBA/H mice		Effectors from Athymic CBA/H nude mice	
		VV-HA-IL2 -immune	VV-HA-TK -immune	VV-HA-IL2 -immune	VV-HA-TK -immune
1	20:1	64	46	62	46
	7:1	53	30	45	27
	2:1	29	19	27	14
2	20:1	61	39	56	50
	7:1	37	20	43	30
	2:1	22	8	32	13
3	20:1	54	45	56	48
	7:1	44	24	43	25
	2:1	25	11	32	14
6	20:1	12	15	63	40
	7:1	6	11	39	20
	2:1	3	7	26	13
Unimmunized controls	20:1	28		43	
	7:1	13		21	
	2:1	6		12	

[a] Nude mice were infected i.v. with 10^6 PFU and normal mice i.p. with 10^7 PFU.

[b] Effector : target ratio.

[c] SEM were less than 5%.

Table 7.

Phenotype of cytotoxic spleen cells from mice immunized with VV-HA-IL2 or VV-HA-TK.

Treatment	E:T [a]	% specific lysis of YAC-1 targets [b]					
		Euthymic CBA/H Splenocytes [c]			Athymic CBA Nude Splenocytes [d]		
		Unimmunized Control	VV-HA-IL2 -immune	VV-HA-TK -immune	Unimminized Control	VV-HA-IL2 -immune	VV-HA-TK -immune
C only	20:1	26	75	39	43	67	54
	7:1	11	36	16	21	47	28
	2:1	7	17	10	12	26	15
C + anti-	20:1	25	38	30	39	42	43
Thy-1.2	7:1	10	19	10	19	23	21
	2:1	7	8	6	11	16	12
	20:1	1	25	6	8	20	9
C+anti-as-GM_1 [e]	7:1	0	13	4	4	8	3
	2:1	0	3	0	2	3	0
	20:1	31	69	32	40	66	49
C+anti-CD8	7:1	14	32	17	23	44	23
	2:1	9	15	9	11	29	18
	20:1	25	67	37	41	63	51
C+anti-CD4	7:1	12	30	18	23	45	24
	2:1	8	18	9	9	24	10

Table 7 cont.

[a] Effector : target ratio.

[b]Results shown are representative of 3 separate experiments. Assays were done in triplicate for each effector : target ratio and standard errors of means (SEM) were lower than 5% and are omitted for clarity.

[c] Spleens harvested 3 days after immunization i.p. with 10^7 PFU of virus.

[d] Spleens harvested 3 days after immunization i.p. with 10^6 PFU of virus.

[e] The lysis of YAC-1 targets by VV-HA-IL2-induced effectors in nude or normal mice could be further reduced by increasing the concentration of anti-as-GM_1 antisera (used in the above experiment at a 1:50 dilution) to a 1:10 dilution.

Table 8.

Resitance to lysis by complement and antiserum treatment by VV-HA-IL2-induced cytolytic effectors can be overcome with increasing concentration of anti-as-GM_1.

Treatment [a] (antiserum dilution)	E:T [b]	% specific lysis [c]		
		Splenocytes from [d]		
		3-day-immune normal CBA/H	3-day-immune outbred nude	5-day-immune outbred nude
C only	20:1	49	56	66
	7:1	28	31	44
	2:1	13	17	21
C + anti-as-GM_1	20:1	31	40	49
(1:50)	7:1	15	20	25
	2:1	8	12	15
C + anti-as-GM_1	20:1	17	23	28
(1:25)	7:1	9	11	17
	2:1	4	6	8
C + anti-as-GM_1	20:1	8	13	20
(1:10)	7:1	3	5	9
	2:1	1	3	5

[a] Six day immune splenocytes were treated with antibody + C as described in Materials and Methods.

[b] Effector : target ratio.

[c] SEM were less than 5%.

[d] Splenocytes were obtained from female CBA/H or outbred nude mice that had been infected i.v. with 10^7 PFU of VV-HA-TK or VV-HA-IL2.

Table 9.

Lysis of YAC-1 targets by spleen cells from outbred nude mice infected with VV-HA-IL2 or VV-HA-TK.

Day(s) after Immunization [a]	E:T [b]	% specific lysis [c]	
		VV-HA-IL2-immune effectors	VV-HA-TK-immune effectors
1	20:1	39	28
	7:1	22	12
	2:1	9	5
2	20:1	48	33
	7:1	27	15
	2:1	12	6
3	20:1	54	36
	7:1	34	19
	2:1	16	9
4	20:1	58	34
	7:1	39	18
	2:1	18	7
5	20:1	65	28
	7:1	48	14
	2:1	20	9
6	20:1	62	31
	7:1	45	13
	2:1	17	6
8	20:1	56	25
	7:1	35	11
	2:1	13	4
Unimmunized control	20:1	22	
	7:1	9	
	2:1	3	

Table 9 cont.

[a] Six- to 8-week old female outbred nude mice were infected i.v. with 10^7 PFU of VV-HA-IL2 or VV-HA-TK.

[b] Effector :target ratio.

[c] SEM were less than 5%.

Table 10.

Phenotype of 5-day immune splenocyte effectors from nude mice infected with VV-HA-IL2 or VV-HA-TK.

Treatment [a]	E:T [b]	% specific lysis [c]			
		3-day immune splenocytes [d]		5-day immune splenocytes [e]	
		VV-HA-IL2	VV-HA-TK	VV-HA-IL2	VV-HA-TK
C only	20:1	56	29	68	36
	7:1	31	18	45	18
	2:1	17	8	23	9
C + anti-Thy-1.2	20:1	30	20	36	23
	7:1	14	11	19	12
	2:1	6	4	8	5
C + anti-as-GM_1	20:1	13	3	20	5
	7:1	5	0	9	2
	2:1	3	0	5	0
C + anti-CD4	20:1	59	30	70	35
	7:1	32	16	46	16
	2:1	15	7	25	9
C + anti-CD8	20:1	55	28	65	33
	7:1	30	17	43	17
	2:1	17	7	20	8
C + anti-Thy-1.2 + anti-as-GM_1	20:1	9	1	15	3
	7:1	4	0	7	1
	2:1	2	0	3	0

Table 10 cont.

[a] Splenocytes were treated with antibody + C as described in Materials and Methods. Anti-as-GM_1 antisera was used at a dilution of 1:25.

[b] Effector : target ratio.

[c] SEM were less than 5%.

[d,e] Splenocytes were obtained from 3- and 5-day immune female nude mice that had been infected with 10^7PFU of VV-HA-IL2 or VV-HA-TK i.v.

CHAPTER 4

Analysis of the role of NK cells in rapid viral clearance using NK-cell deficient mice

INTRODUCTION

Host defense mechanisms, both specific and non-specific, have been implicated as antiviral effectors in protection and recovery from infection but the relative importance of any particular mechanism appears to depend on the type of virus, immune status of the host and the severity of infection (reviewed in Chapter 1). The non-specific effectors like macrophages, IFNs and NK cells limit spread of virus early during infection before the induction of specific immune responses like antibody and effector T cells. While macrophages and IFNs have known antiviral functions (Zisman *et al.* 1970; Blanden, 1971b; Gresser *et al.*, 1976) the role of NK cells has remained obscure. However, recent reports have provided conclusive evidence for a role of NK cells in mediating protection against development of fatal viral disease (Rager-Zisman *et al.*, 1987) and limiting the severity and duration of acute viral infection (Bancroft *et al.*, 1981; Shellam *et al.*, 1981; Bukowski *et al.*, 1983; Bukowski *et al.*, 1984). The relative importance of NK cells in contributing to antiviral mechanisms during some viral infections was demonstrated in the above studies by the use of specific antisera to deplete NK cells, adoptive transfer of NK cells or by the use of NK cell deficient beige mice.

The results presented in Chapter 3 indicated a possible role for NK cell-mediated antiviral mechanism(s) in contributing to the rapid clearance of VV-HA-IL2 in normal and nude mice. However, a causal relationship between virus-encoded IL-2-activated NK cells and antiviral function *in vivo* was not established. Moreover, it was also shown that not all the cytolytic activity on YAC-1 targets by splenocytes from VV-HA-IL2-infected mice was mediated by as-GM_1^+ NK cells.

The present study was designed to investigate the causal relationship between the IL-2 induced NK cell activation and antiviral effects *in vivo* using NK deficient mouse models. Nude or normal mice, depleted of NK cells using specific antisera/Mab, and NK deficient beige mice were inoculated with the IL-2-encoding or control viruses to study the immune responses and viral replication.

MATERIALS AND METHODS

Mice.

Beige mice of the CBA background (Cabg/bg) were purchased from the Animal Resources Centre, Perth. Normal CBA/H and C57BL/6 mice and athymic Swiss outbred mice were obtained from the Animal Services Section of the John Curtin School of Medical Research. All mice, bred under specific pathogen free conditions, were used between 6-10 weeks of age and in individual experiments age- and sex-matched mice were used.

Viruses.

As in Chapter 2.

Antisera.

The following antisera were used at the indicated dilutions for cell surface analysis by cytofluorometry: anti-as-GM_1 at a 1:20 dilution, F4/80, which is a rat-antimouse-macrophage Mab (Austyn and Gordon, 1981), kindly provided by Dr. W. Allen (JCSMR) as culture supernatant, was used undiluted. Biotinylated anti-Thy-1.2 (Becton Dickson, Mountain View, CA, USA) at a 1:1000 dilution, anti-CD4 and anti-CD8 (both described in Chapter 3) and anti-NK-1.1, clone PK136 (Koo and Peppard, 1984; Koo *et al.*, 1986) obtained from the American Type Culture Collection (Rockville, MD, USA) were used as undiluted culture supernatants. FITC-conjugated sheep anti-rabbit IgG, sheep anti-rat IgG and sheep anti-mouse IgG (Silenus Laboratories, Victoria, Australia) at 1:20 dilutions and streptavidin R-phycoerythrin conjugate (Serotec Ltd., Blackthorn Bicester, England) at a 1:30 dilution were all used as secondary antibodies. Sheep IgG (Silenus Laboratories) was used at a 1:20 dilution to block Fc receptors. The working dilutions of all the above reagents were chosen from staining titration data.

In vitro cell depletion with antibody and complement.

The panel of antisera/Mab used and the method for cell depletion *in vitro* has been described in Chapter 3.

***In vivo* NK cell depletion in nude mice.**

Outbred Swiss nude mice were depleted of NK (as-GM_1^+) cells *in vivo* using the method described by Habu *et al.* (1981) with modifications. Anti-as-GM_1 was administered i.v. followed by the i.n. route after light anaesthesia with ether, to effectively deplete NK cells and to promote lung VV titres. The procedure involving antibody administration via two routes is based on the findings of Stein-Streilein and Guffee (1986), that treatment with anti-as-GM_1 by the two routes was superior to the i.v. route alone. This was particularly essential for the depletion of NK cells in the lung. Stock antiserum was diluted 1:5 in PBS prior to use. Mice were given 150 µl of antiserum i.v. and 50 µl i.n. on days -5, -3, -1, 0, 1, and 2 where day 0 was the day of infection with virus. Control mice were similarly treated with PBS or normal rabbit serum (NRS). Treatment was continued everyday after the day of infection since preliminary depletion experiments showed the rapid reemergence of as-GM_1^+ cells (assessed by flow cytometry and lysis of YAC-1 targets) in spleens of mice infected with VV-HA-IL2. Anti-as-GM_1 antiserum treatment does not deplete as-GM_1^- NK cell precursors and IL-2 is known to give rise to as-GM_1^+ cells from precursors which lack the marker.(Hasui *et al.*, 1989).

A Mab that recognizes the NK-1.1 marker (only expressed by some strains of mice) on murine NK cells was available but appropriate C57BL/6 nude mice were not available. Therefore, the use of the NK-1.1$^-$ nude mouse model for this study was not possible.

Depletion of NK cells in normal mice.

Although the as-GM_1 marker is expressed predominantly on NK cells in normal mice, there have been reports that it is also expressed on some T cells (Suttles *et al.*, 1986) and macrophages (Wiltrout *et al.*, 1985). Since as-GM_1 was also found on murine ovarian cells (unpublished observations; see Chapter 6), it was not possible to demonstrate the effect of as-GM_1^+ cell depletion on VV replication in ovaries. Therefore, NK cell depletion in normal mice was achieved by the use of the anti-NK-1.1 Mab (Koo *et al.*, 1986), which recognizes the NK-1.1 marker. C57BL/6J mice which express this marker were selectively depleted of NK cells using a modified method, originally described by Koo *et al.* (1986). Female C57BL/6J mice aged 5-6 weeks were given approximately 1 mg antibody in 0.2 ml of PBS i.v. and 250 µg in 50 µl of PBS i.n. at 7 day intervals over a

three week period. Three days after the last treatment, mice were given the antibody again 1 day prior to immunization with virus. Antibody treatment was continued on the day of infection and everyday thereafter except on the day the mice were sacrificed. Efficiency of the depletion was assessed by cytolytic activity of the splenocytes on YAC-1 targets and flow cytometry.

Fluorescent staining of cells for flow cytometry.

For single colour staining, aliquots of cells ($1x10^6$ splenocytes or $3x10^5$ leucocytes in complete EMEM) that had been depleted of erythrocytes and B cells (see below), were pelleted by gentle centrifugation, the medium removed and cells were resuspended in 100 µl of Mab appropriately diluted in EMEM or undiluted and incubated for 30 min at 4°C. Samples were centrifuged through 0.5 ml of FCS and resuspended in 100 µl of the appropriate second stage reagent and incubated for a further 30 min at 4°C. After a final centrifugation through FCS, samples were resuspended in 0.5ml of complete EMEM for flow cytometry. Macrophages were incubated with sheep IgG to block Fc receptors prior to primary antibody staining. At times when flow cytometry was performed a day after staining, the cells were fixed in 1% gluteraldehyde in PBS.

Flow Cytometry.

Fluorescent labelled samples were analyzed on a FACS 440 flow cytometer (Becton Dickinson Immunocytometry System, CA, USA) using 100 mW of 488 nm laser light to excite FITC and phycoerythrin (PE). Fluorescence emissions of FITC and PE were detected following passage trough a 560 nm dichroic mirror and a 535/15 nm band pass filter for PE-fluorescence. For single colour analysis, 20,000 events were collected from each sample. Gating of dead cells and red cells was performed using forward light scatter. Analysis of data was performed on a Consort 40 computer (Becton Dickinson) using the DISP4 program. The percentage of positively stained cells was calculated by subtracting control cells stained with the fluorochrome-conjugated reagents, from those stained with primary antibodies and fluorochrome-conjugated reagents.

B cell depletion in splenocyte and liver leukocyte populations.

Splenocyte and liver leukocyte preparations were depleted of B cells before staining for the cell surface analysis by cytofluorimetry. Sheep red blood cells (SRBC) were coupled to sheep anti-mouse Ig in 0.1% $CrCl_3$. B cells were rosetted with sheep anti-mouse Ig-coupled SRBC and removed by Ficoll-Paque separation. This method generally resulted in more than 95% depletion of B cells.

Isolation of leukocytes from ovaries for cytotoxicity assays.

Ovarian effector cells were prepared from pooled ovaries obtained from 30-40 mice per group. Ovaries were carefully minced and incubated in EMEM containing 2mg/ml of collagenase (Boehringer Manheim, Indianapolis, IN., USA) at 37°C for 30 min before gently pressing through a stainless steel mesh. The resulting cell suspension was washed once in EMEM containing 5% heat-inactivated FCS. Enrichment for NK cells was carried out by either passage through nylon wool columns, Ficoll-Paque density gradient separation or Percoll (Pharmacia, Uppsala, Sweden) gradient separation as described elsewhere (Patel and Linna, 1984).

Isolation of liver leukocytes.

Liver leucocytes were isolated from uninfected control or virus infected mice after *in situ* perfusion with enzyme (McIntyre and Welsh, 1986). Mice were anaesthetized with sodium pentobarbital (1mg, i.p.) prior to catheterizing the portal vein with polyethylene tubing. Perfusion (2.5ml/min for 5 min) was carried out initially with Ca^{2+}- and Mg^{2+}- free Hanks balanced salt solution (HBSS) at 37°C to flush peripheral blood from the liver. The perfusate was then changed to prewarmed (37°C) HBSS containing 0.05% (wt/vol) collagenase and perfusion was continued for a further 10 min. After that, the liver was excised and teased apart with fine forceps in HBSS. The resulting suspension was aspirated several times, clumps were removed by passage through nylon mesh and pelleted at 1,000 g. The parenchymal cells were removed by Ficoll-Paque density gradient separation and the leucocytes were removed from the interphase, washed 3 times in EMEM and resuspended in complete EMEM. Liver leucocytes obtained this way were used in cytotoxicity assays and cell surface analysis by cytofluorimetry.

Cytotoxicity assays.

The standard chromium release assay was performed as described in Chapter 3.

In experiments using VV-HA-TK- or VV-HA-IL2-infected targets , L929 cells were infected with either virus for 3 h and then labelled with ^{51}Cr for 1 h, washed 3 times and resuspended in complete EMEM. Samples were assayed in triplicate ($2x10^4$ target cells plus the appropriate number of splenocyte effector cells or $1x10^3$ target cells plus the appropriate number of ovarian or liver effector cells in 200 µl per well) for 4 h with YAC-1 targets, 6 h for L929 and EL4 targets and 8 h for macrophage targets at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Preparation of macrophage targets for cytotoxicity assays.

Resident peritoneal macrophages were isolated from C57BL/6 female mice by peritoneal lavage with ice-cold Pucks A saline. Pooled macrophages were pelleted by centrifugation and resuspended in complete EMEM. The desired number of cells were added to each well and allowed to adhere for 1 h at 37°C after which non-adherent (approximately 50% of the total cells) cells were washed off with medium. Macrophages were labelled with 2 µCi ^{51}Cr per well and simultaneously infected with VV-WR at 40PFU/cell. Control macrophage targets were left uninfected. After a further incubation for 1 h at 37°C, plates were washed 3 times with EMEM before addition of the appropriate number of effectors. Spontaneous ^{51}Cr release for uninfected macrophages was between 10-15% and for infected macrophages was between 18-22%.

Histology.

Organs removed from mice after cervical dislocation were fixed in 10% neutral buffered formalin before being processed for embedding in paraffin. Sections of tissue (4 µm thick) were stained with haematoxylin and eosin and mounted in mounting fluid.

Virus titration.

As in Chapter 2.

RESULTS

The effect of NK (as-GM_1^+) cell depletion in nude mice on recombinant VV replication.

In order to establish that NK (as-GM_1^+) cells contributed to the control of viral replication and recovery from infection with VV-HA-IL2, outbred nude mice were depleted of as-GM_1^+ by repeated injections with anti-as-GM_1 antiserum. The procedure described in Materials and Methods resulted in the elimination of more than 98% of splenic as-GM_1^+ cells, as assessed by flow cytometry and completely abolished the cytolytic activity on YAC-1 targets (data not shown). Control mice were treated using a similar regime with either PBS or normal rabbit serum (NRS) diluted in PBS. Groups of NK cell depleted mice and control mice infected i.v. with 10^7 PFU of VV-HA-IL2 or VV-HA-TK were sacrificed 5 days later for the determination of splenic NK activity (YAC-1 target lysis) and virus titres in organs.

Control nude mice inoculated with VV-HA-IL2 had elevated splenic NK activity which was about 3-fold higher than the activity induced by VV-HA-TK, 3 and 5 days after infection as described in Chapter 3 (data not shown). Splenocytes obtained from virus infected mice which had been treated with anti-as-GM_1 antiserum had virtually no cytolytic activity on YAC-1 or L929 targets (data not shown).

NK cell depletion in nude mice infected with VV-HA-TK resulted only in marginal and insignificant ($p>0.05$) increases in the mean viral titres of ovaries and lungs, 3 and 5 days after infection compared to control mice (Table 1). On the other hand, depletion of NK cells had marked effects on VV-HA-IL2 replication in lungs and to a lesser extent in ovaries of nude mice. Three days after infection, the mean lung VV-HA-IL2 titre increased by 1.2 $\log_{10}$ and the ovarian titre was higher by 0.8 $\log_{10}$ and, by 5 days after infection, the titres increased by 1.6 $\log_{10}$ and 1.0 $\log_{10}$ respectively, compared to control mice. Despite a satisfactory depletion of NK cells, ovarian VV-HA-IL2 titres were never as high as levels of VV-HA-TK. In contrast, VV-HA-IL2 titres in lungs of NK cell-depleted mice attained levels that were similar to the mean lung VV-HA-TK titre in control mice. These results demonstrated that the strong NK response induced by VV-HA-IL2 played an important role in control of virus replication in ovaries and lungs. In addition, they implied that some

other mechanism(s), independent of NK cell-mediated cytotoxicity, may be involved in viral clearance in infected ovaries. Although depletion of the as-GM_1^+ NK cells did not promote VV-HA-TK replication to a statistically significant extent ($p>0.05$), increased viral titres were apparent.

Separate groups of nude mice that were infected with 10^7 PFU of VV-HA-IL2 or VV-HA-TK and treated with anti-as-GM_1 antiserum, were monitored for signs of morbidity and mortality. All mice that were infected with VV-HA-TK died within 14 days after infection, and NK cell depletion did not accelerate the MTD (Table 2). In contrast, no mortality was recorded in NK cell-depleted nude mice that had been infected with VV-HA-IL2 although morbidity was noted.

NK cell depletion in normal mice : Effect on YAC-1 target lysis and viral replication.

The procedure described for NK cell depletion with the NK-1.1 Mab was very efficient since the cytolytic activity of normal splenocytes on YAC-1 targets was completely abolished (Fig. 1) and NK-1.1^+ cells were absent from the C57BL/6 splenocyte population when assessed by flow cytometry (data not shown). Lysis of YAC-1 targets by 3-day immune effectors from VV-HA-IL2-infected mice was about 3-fold higher than the VV-HA-TK-induced splenic NK activity. Treatment with the Mab in mice infected with VV-HA-TK resulted in a 9-fold reduction in levels of YAC-1 target lysis (Fig. 1) and was comparable with levels of lysis mediated by unimmunized controls. On the other hand, despite a 9-fold reduction in levels of NK activity in mice given Mab and VV-HA-IL2, levels of cytolytic activity above control levels was still present (Fig. 1) although no NK-1.1^+ cells could be detected by flow cytometry. This cytolytic activity could have been due to some other category of effectors which were presumably expanded in the presence of IL-2.

Ovaries and lungs from groups of 4 mice that had been treated or otherwise with the NK-1.1 Mab and infected with 10^7 PFU virus i.v. 3 days previously were used to determine viral titres. Titres of VV-HA-TK in ovaries and lungs of NK cell-depleted mice were increased marginally above levels in control mice but the increases were statistically insignificant ($p>0.05$) compared to mean lung and ovarian titres in control mice (Table 3). In contrast, the mean VV-HA-IL2 titre was more than 2 $\log_{10}$ PFU higher in ovaries and increased by 1.3 $\log_{10}$ PFU in

lungs of Mab-treated mice in comparison with untreated controls. However, the increased VV-HA-IL2 titres in ovaries of NK cell-depleted mice were not elevated to the level of ovarian VV-HA-TK titres. While it is likely that activated NK cells could have been largely responsible for the clearance of VV-HA-IL2 from the lungs of normal mice, other factors besides NK cells appear to have been involved in viral clearance in ovaries. Although no evidence has been obtained, the non-NK cytolytic effectors (obtained from NK cell-depleted, VV-HA-IL2-infected mice) (Fig. 2) are candidates for further investigations.

The replication of VV-HA-IL2 in CBA/H mice (non-NK-1.1 expressing strain) was not significantly ($p>0.05$) affected after treatment with the NK-1.1 Mab (Table 3).

Tc cell responses in NK cell-depleted mice.

The data presented above established that NK cell depletion resulted in enhanced VV-HA-IL2 replication. It may be expected that the Tc cell response in these mice would be elevated due to the increased viral antigen load. The lysis of VV-WR-infected C57BL/6 macrophages 6 days after infection in NK cell-depleted C57BL/6 mice was almost 3-fold higher than in control mice (Table 4) and the lysis of uninfected, syngeneic macrophage targets was insignificant. Interestingly, the cytolytic effectors obtained from antibody-treated mice exhibited high levels of lysis of uninfected H-2-compatible EL-4 targets whereas the lysis by effectors from untreated mice was minimal (Table 4). This finding suggested further that the depletion of NK cells resulted in the emergence of at least one other cytolytic population that was presumably expanded and/or activated in the presence of IL2.

Although no virus was recovered from the lungs of antibody treated or untreated mice, about 10^4 PFU of virus was recovered from ovaries of mice depleted of NK cells 6 days after infection (Table 4). VV-HA-IL2 was not recovered from ovaries of untreated mice.

Studies with beige mice.

Beige mice lack functional cytolytic NK cells (Roder and Duwe, 1979). They possess the phenotypically "NK-like" cells which are defective at the level of postbinding trigger of target cell lysis. The relative importance of

cytolytic NK cells, especially in VV-HA-IL2 clearance, was investigated using beige mice since NK cells are thought to mediate antiviral activity by direct cytolysis of virus infected cells as well as by the secretion of antiviral factors. NK and Tc cell responses as well as viral replication kinetics were compared in beige and normal CBA mice following infection with VV-HA-TK or VV-HA-IL2.

Comparison of NK and Tc cell responses in beige and normal CBA mice.

Groups of 4 age-matched female CBA normal and beige mice were infected i.v. with 10^7 PFU of VV-HA-TK or VV-HA-IL2. On days 1, 2, 3, 4, 6 and 9 after infection, splenocytes were tested for NK and Tc activity while ovaries were used to measure virus titres.

Splenocytes obtained from VV-HA-IL2 or VV-HA-TK-infected beige mice had no cytolytic activity on YAC-1 targets on any of the days after infection (data not shown). Cytolytic activity on the classical NK targets observed using effectors from 2-day immune beige and normal mice is shown in Table 5. While VV-HA-IL2 induced about a 3-fold higher splenic NK activity above the VV-HA-TK induced response in normal mice, the IL-2-encoding virus did not induce any such activity in beige mice.

The kinetics of Tc cell generation in beige and normal mice after inoculation with VV-HA-TK or VV-HA-IL2 were similar although not identical (data not shown). Peak responses were observed using 6 day immune splenocytes (Table 6). Tc cell activity induced by either virus in beige mice was slightly higher than activity in normal mice 6 days post infection (Table 6).

Comparison of the kinetics of viral replication in beige and normal mice.

One day after infection with 10^7 PFU virus, titres of VV-HA-TK and VV-HA-IL2 in ovaries of beige and normal mice were comparable but from the second day onwards, VV-HA-TK titres were always higher than VV-HA-IL2 in both beige and normal mice (Figure 2). This difference was more marked in normal mice, VV-HA-IL2 titres reached a peak of 5.6 $\log_{10}$ PFU on day 2 and declined to become undetectable by day 6. In beige mice VV-HA-IL2 titres peaked at 6.4 $\log_{10}$ on day 4 and declined thereafter, but 4.8 $\log_{10}$ persisted on day 6 and 2.0 $\log_{10}$ remained on day 9.

The control virus, VV-HA-TK, behaved similarly (though not identically) in beige and normal mice, reached a peak of around 8 $\log_{10}$ on days 3 and 4 and declined to about 4 $\log_{10}$ on day 9, but clearance of virus was slightly delayed in beige mice compared to normal mice, after peak titres were reached.

The above data is compatible with the idea that a strong NK cell response induced by VV-HA-IL2 contributed to rapid clearance of this virus from ovaries of normal mice. Nevertheless, beige mice clearly derived benefit from IL-2, even though they produced no detectable cytolytic NK activity in spleens. Since the generation of Tc cell response was similar to normal mice, this benefit was presumably mediated by mechanisms as yet undefined, possibly by activation of the NK-like cells to secrete cytokines which contributed to antiviral mechanisms.

NK cells lyse VV-HA-IL2-infected L929 cells more efficiently than VV-HA-TK-infected cells.

Data from the above experiments established that depletion of NK cells (as-GM_1 antiserum or NK-1.1 Mab treatment) or a lack of cytolytic NK cells (beige mice) resulted in enhanced VV-HA-IL2 replication. NK cells could contribute to control of vaccinia replication *in vivo* either by lysing infected cells or by secreting anti-viral factors. NK cells were able to lyse vaccinia-infected L929 cells (Chapter 3, Tables 1 and 3). They also killed VV-HA-IL2-infected targets more efficiently than VV-HA-TK-infected targets (Table 7). The mechanism of enhanced lysis could be due to augmentation of NK cell activity by IL-2 secreted by the VV-HA-IL2-infected L929 cells during the 6 h ^{51}Cr release assay. Addition of exogenous recombinant murine IL-2 to the NK assay or pulsing effector cell suspensions with IL-2 for 1-4 h before the cytotoxicity assay also resulted in enhanced lysis of L929 or YAC-1 targets (data not shown). The enhanced killing was not due to increased target cell susceptibility to lysis since pulsing target cells with IL-2 prior to the assay did not affect the levels of lysis (data not shown).

Liver lesions caused by VV-HA-IL2 contain more infiltrating mononuclear cells than lesions caused by VV-HA-TK.

The above data suggest that NK cells could contribute to inhibition of virus replication *in vivo* by lysing infected cells, providing that they were present at the site of infection. The recent report (Natuk and Welsh, 1987) that recombinant human IL-2 is chemotactic for murine large granular lymphocytes, including NK cells, suggests a mechanism whereby this could occur. Therefore, the possibility that infected foci produced by VV-HA-IL2 may attract NK cells more efficiently than VV-HA-TK was investigated by histologically examining liver lesions of CBA/H mice 12, 24, 48 and 72 h after i.v. infection with 10^7 PFU virus. The lesions caused by VV-HA-IL2 (Figure 3a) consistently contained more infiltrating mononuclear cells than lesions caused by VV-HA-TK (Figure 3b). Similar observations were also made in athymic nude mice 3, 6, 9 and 12 days after i.v. infection with 10^7 PFU of VV-HA-TK or VV-HA-IL2 (data not shown)

Phenotypic analysis of liver leukocytes.

Cytofluorimetric analysis of liver leukocytes isolated from euthymic CBA/H mice 3 days after infection revealed that there were more as-GM_1^+ and Thy-1.2$^+$ cells in the infiltrate caused by VV-HA-IL2 than that due to VV-HA-TK (Table 8). In addition, the liver infiltrates caused by both viruses also contained macrophages (F4/80$^+$ cells), CD4$^+$ and CD8$^+$ T cells. In athymic nude mice, infiltrates caused by VV-HA-IL2 also contained more as-GM_1^+ and Thy-1.2$^+$ cells than VV-HA-TK induced infiltrates. However, no CD4$^+$ or CD8$^+$ T cells were detected in liver infiltrates of VV-HA-TK and VV-HA-IL2-infected nude mice which otherwise contained macrophages.

Cytolytic activity of liver leukocytes.

The ability of liver leucocytes to lyse the NK sensitive YAC-1 targets was tested. Liver leucocyte effectors obtained from either normal CBA/H or athymic Swiss outbred nude mice 3 days after i.v. infection with 10^7 PFU of VV-HA-TK or VV-HA-IL2 lysed YAC-1 targets (Fig. 4). Lysis was consistently higher using effector cells from VV-HA-IL2-infected mice than from VV-HA-TK-infected mice but the difference was more marked in nude mice than in normal mice.

Isolation of leukocytes from ovaries of VV infected mice:

Functional and phenotypic characterization.

In Chapter 2, data on VV replication in mouse ovaries was presented. While VV-HA-TK grew to between 10^8-10^9 PFU in ovaries of normal CBA/H of Swiss outbred nude mice, VV-HA-IL2 was rapidly cleared (Chapter 2, Figures 3 and 4). The highly productive infection made the ovary a potentially sensitive indicator of changes in the virus-host relationship caused by IL-2. Histological examination of infected ovaries (Chapter 6, Figure 1b) suggested that VV-HA-TK replicated rapidly in ovarian stroma and destroyed many stromal cells by day 3 after infection. Damage caused by VV-HA-IL2 was barely detectable (Chapter 6, Figure 1a), consistent with the low levels of virus recovered.

Since IL-2 does not directly inhibit virus replication *in vitro* (unpublished observations), the inhibition of VV-HA-IL2 replication in ovaries of infected mice could be attributed in part to local cell-mediated mechanisms induced by the expression of this lymphokine in foci of infection. The nature of VV-induced histopathology in ovarian stroma did not permit clear evaluation of inflammatory infiltration as was possible in the liver, therefore attempts were made to isolate and characterize infiltrating leukocytes from ovaries of VV-infected mice. Leukocytes were isolated from ovaries of 30 mice per group which had been infected i.v. with 10^7 PFU of VV-HA-TK or VV-HA-IL2, 3 days previously and enriched for NK cells by Ficoll-Paque density gradient separation, Percoll density gradient separation or by passage through nylon wool columns.

Irrespective of the method used for enrichment, ovarian effector cells obtained from mice infected with VV-HA-IL2 had a 9-fold greater cytolytic activity on YAC-1 targets (Table 9) than cells induced by VV-HA-TK. The ovarian effector cells mediating lysis of YAC-1 targets were Thy-1.2$^{\pm}$, as-GM_1^+, CD4$^-$ and CD8$^-$ (Table 10). Treatment of VV-HA-IL2-induced ovarian cytolytic cells with anti-as-GM_1 and C completely abrogated the capacity for lysis of YAC-1 targets, but the lysis of L929 targets was only partially reduced. Furthermore, treatment of these effectors with anti-Thy-1.2 and C did not abrogate the lytic activity on L929 targets. This suggested the existence of two different populations of effector cells, one

being as-GM_1^+ and largely Thy-1.2^+ which could lyse both YAC-1 and L929 targets, and a second being as-GM_1^- and Thy-1.2^- which could lyse only L929 targets. Whether these as-$GM1^-$ and Thy-1.2^- cytolytic effectors are mutually exclusive is not known.

Interestingly, no NK activity could be demonstrated in ovaries of VV-HA-IL2-infected beige mice (data not shown). The clearance of VV-HA-IL2 from ovaries of these mice was delayed in comparison with clearance from ovaries of normal mice (Fig. 2).

DISCUSSION

The evidence presented earlier unequivocally demonstrated a crucial role for IL-2-activated NK cells in mediating antiviral mechanisms. Depletion of NK cells in nude or normal mice resulted in enhanced VV-HA-IL2 titres but increases in VV-HA-TK titres were marginal (Tables 1 and 3), suggesting that NK cells were probably not an obligatory requirement for the control of VV-HA-TK growth early during the course of infection. This finding, however, is not consistent with a previous report (Bukowski *et al.*, 1983) which indicated that the wild type virus, VV-WR, replicated to significantly higher levels in spleens and livers of NK cell-depleted normal mice. The reduced virulence of VV-HA-TK compared to VV-WR (Chapter 2, Table 1), and the inability of VV-HA-TK to replicate as efficiently as the wild type virus in spleens or livers (Chapter 2, Fig. 4 and Chapter 6, Table 1) of normal mice may have contributed to the difference.

Comparison of viral replication kinetics in ovaries of beige and normal mice suggested that the elevated NK cytolytic activity induced by VV-HA-IL2 in normal mice, but not in beige animals, may have made a significant contribution to control of VV replication in ovaries. Whereas normal mice were able to clear VV-HA-IL2 by the sixth day after infection, virus persisted until at least day 9 post-infection in ovaries of beige mice. This data is consistent with a role for NK-cell-mediated cytolytic activity in viral clearance.

Nonetheless, beige mice did clearly benefit from virus encoded IL-2 because VV-HA-IL2 titres were consistently lower than control VV-HA-TK throughout the course of infection from days 2-9. This implied that some other mechanism, independent of NK cell-mediated cytolytic activity, also contributed to clearance of VV-HA-IL2 in beige mice. Since the defect in the NK cells of beige mice is limited to an absence of cytolytic activity (Roder and Duwe, 1979), it remains possible that the NK-like cells in these animals helped to clear infection by release of antiviral factors such as IFN-γ or TNF-α which are known to be triggered by IL-2. Apart from a lack of cytolytic NK cells, beige mice are reported to have both defective macrophage-mediated anti-tumour activity (Mahony *et al.*, 1980) and alloreactive Tc cell responses (Saxena *et al.*, 1980). Data presented in this Chapter clearly established that beige could mount strong anti-VV Tc cell response. However, it is not clear if the defective macrophage-

mediated anti-tumour activity of beige mice may extend to antiviral activity.

Absence of cytolytic NK cells in beige mice or the depletion of NK cells in normal mice resulted in enhanced Tc cell responses to VV-HA-IL2, compared to control normal mice. While the enhanced response, presumably due to an increase in viral antigen load, is to be expected, depletion of NK cells in normal mice also resulted in the emergence of an unidentified population of cytolytic effectors which efficiently lysed uninfected EL-4 targets 6 days after infection. In addition, VV-HA-IL2 but not VV-HA-TK, induced cytolytic cells in NK cell-depleted C57BL/6J mice which had substantial levels of lysis of YAC-1 targets 3 days post-infection. It is relevant to note that, in the presence of IL-2, spleen- and liver-derived macrophage precursors develop into typical granula-containing cells *in vitro* and acquire the capability to lyse YAC-1 targets (Baccarini et al., 1988). While the antiviral efficacy of these non-NK effectors is not known, the data suggests that virus-encoded IL-2 predominantly activates NK cells, but in their absence, other IL-2 responsive lymphoid (or myeloid) cells are activated.

In order for NK cells to mediate antiviral activity, their presence within foci of infection may be essential. This would allow their cytolytic activity to operate against virus-infected cells, thus preventing further viral replication in those cells. It would also result in local production of potentially effective concentrations of antiviral factors. NK activity was demonstrated in the ovary and liver. Elevated NK activity coincided with the rapid clearance of VV-HA-IL2 from ovaries of infected normal mice but not from ovaries of beige mice which had no detectable NK activity in ovaries or spleens. Clearance of VV-HA-TK which induced only low levels of NK activity in ovaries of normal mice was significantly delayed compared to VV-HA-IL2. Furthermore, histology of infected livers indicated that VV-HA-IL2 induced more rapid mononuclear cell infiltration into foci of infection than VV-HA-TK. Infectious foci due to VV-HA-IL2 also contained more mononuclear cells which included NK cells as well. These results are consistent with the idea that NK cells are attracted to foci of infection and that IL-2 may be an important chemotactic influence (Natuk and Welsh, 1987).

Since VV-infected L929 targets were lysed *in vitro* by the NK-like activity generated during infection *in vivo*, it is possible that virus-infected cells are subjected to NK-mediated lysis during infection *in vivo*. The finding that VV-HA-IL2 infected targets were lysed more efficiently than control virus infected targets suggests further that NK cells attracted to foci of infection may be activated by the virus-encoded IL-2, hence resulting in efficient viral clearance.

While an important role for NK cells in contributing to control of VV-HA-IL2 replication has been established above, there is sufficient evidence which suggests that non-NK-mediated antiviral mechanisms are also involved. Firstly, NK cell-deficient beige mice cleared VV-HA-IL2 more efficiently than VV-HA-TK as discussed earlier. These mice derived benefit from virus-encoded IL-2, despite the lack of cytolytic NK cells. Secondly, at least two distinct populations of cytolytic effector cells were isolated from ovaries of VV-HA-IL2-infected normal mice. The first had a phenotype of NK cells and was as-GM_1^+, Thy-1.2$^\pm$, $CD4^-$ and $CD8^-$. The second population lacked any of the above markers. It appears very likely that both these populations contributed to antiviral mechanisms *in vivo*. This is because titres of VV-HA-IL2 in ovaries of NK cell-depleted normal mice were always lower than titres of VV-HA-TK in control or NK cell-depleted mice, although VV-HA-IL2 titres increased 100-fold in ovaries of NK cell depleted mice.

Finally, although as-GM_1^+ NK cell-depletion in nude mice resulted in enhanced VV-HA-IL2 replication, no mortality was recorded. While this finding may be further construed as supportive of the above contention that non-NK mediated antiviral mechanisms are also important, survival of NK cell-depleted nude mice from infection with VV-HA-IL2 may have been a result of other contributory factors. It is possible that compensation for NK cell depletion may have occurred by the emergence or expansion of other cell types with antiviral activity, especially after exposure to IL-2. IL-2 is also known to induce the maturation of as-GM_1^+ cells from as-GM_1^- progenitors (Hasui *et al.*, 1989), indicating the possibility that as-GM_1^+ cells can re-emerge in NK cell depleted nude mice after infection with VV-HA-IL2.

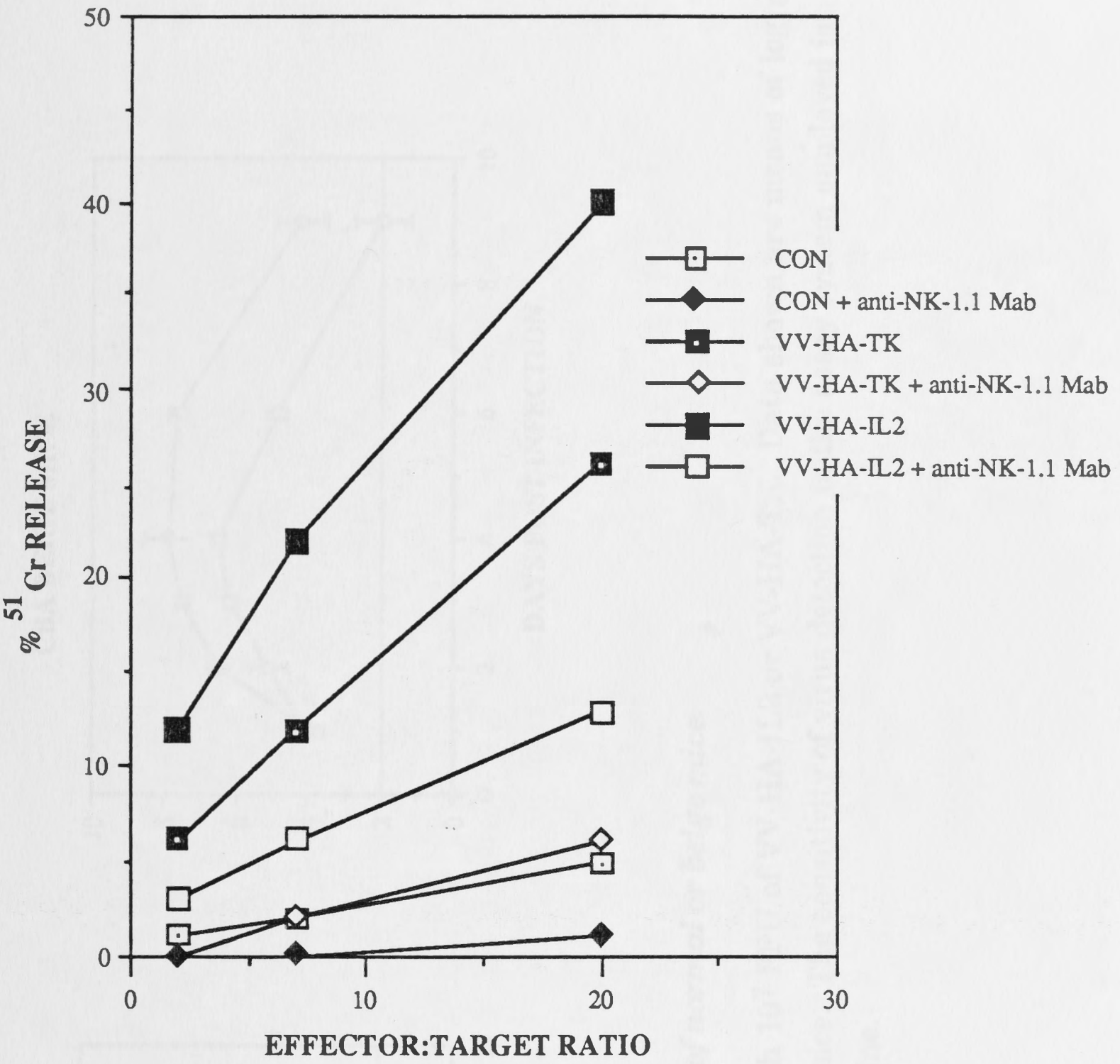


Figure 1. ***NK cell depletion and YAC-1 target lysis.***

C57BL/6J mice were treated with anti-NK1.1 Mab as described earlier and infected i.v. with 10^7 PFU of VV-HA-IL2 or VV-HA-TK. Control mice treated with only PBS were given the similar dose of virus or left uninfected. The capacity for splenocytes from Mab-treated or untreated mice and infected with virus or otherwise to lyse YAC-1 targets was tested 3 days after infection.

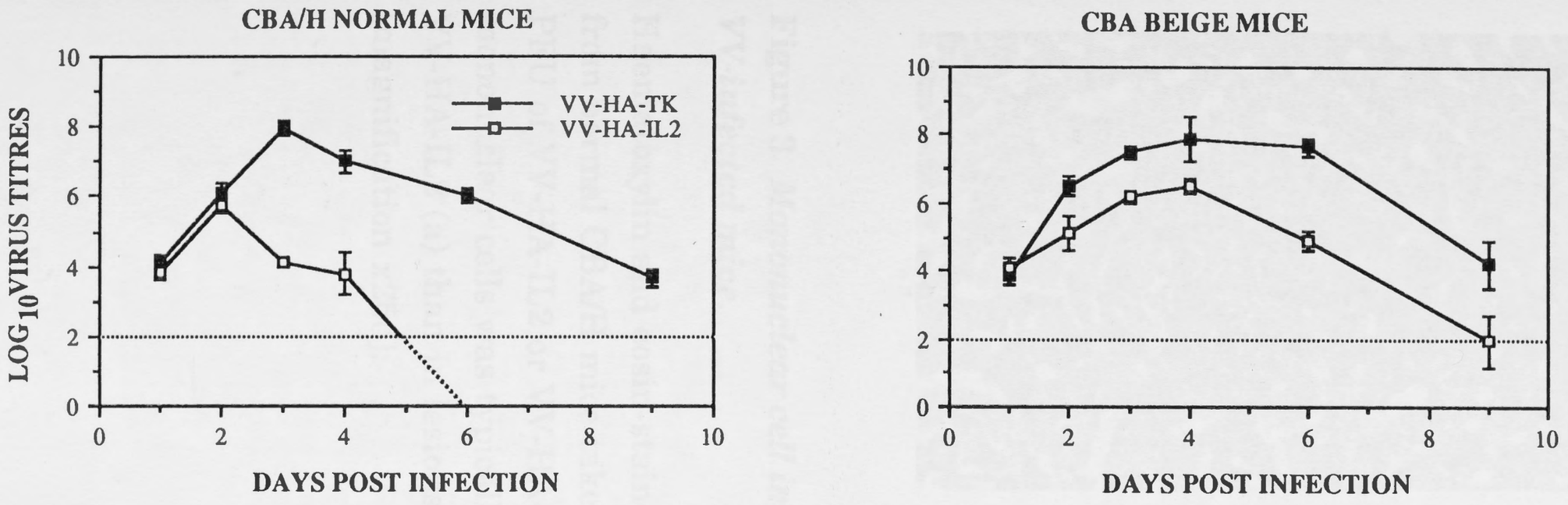


Figure 2. ***Kinetics of virus replication in ovaries of normal or beige mice.***

Normal and beige CBA mice were infected i.v. with 10^7 PFU of VV-HA-IL2 or VV-HA-TK. Data shown are means of $\log_{10}$ PFU/pair of ovaries ± SEM in groups of 4 female mice. The sensitivity of virus detection in the assay system employed is above 100 PFU indicated by the horizontal dotted line.

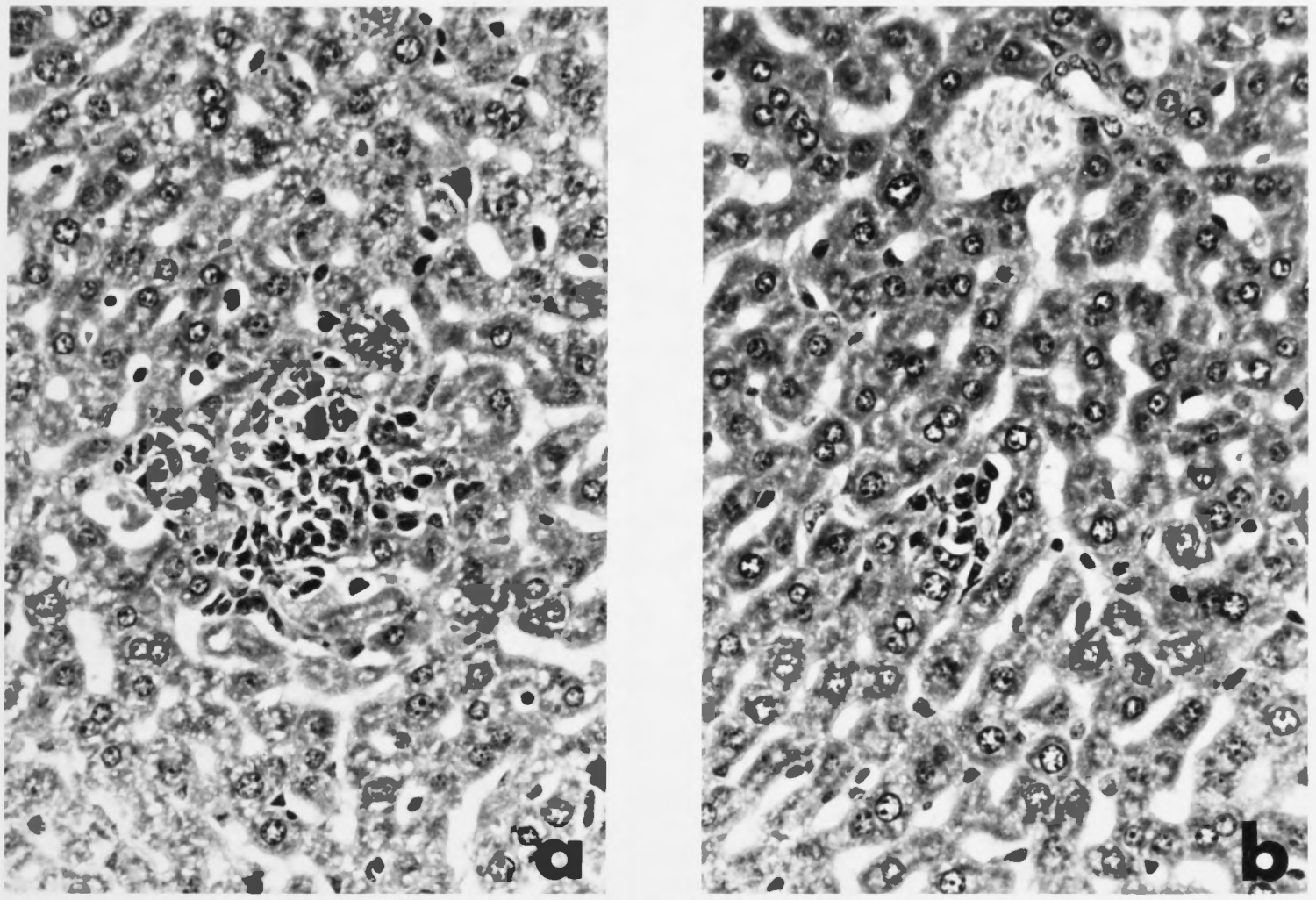


Figure 3. ***Mononuclear cell infiltration in livers of recombinant VV-infected mice.***

Haematoxylin and eosin-stained histological sections of livers from normal CBA/H mice taken 72 h after i.p. injection with 10^7 PFU of VV-HA-IL2 or VV-HA-TK. More infiltration with mononuclear cells was typically seen in liver lesions caused by VV-HA-IL2 (a) than in lesions caused caused by VV-HA-TK (b) [magnification x250].

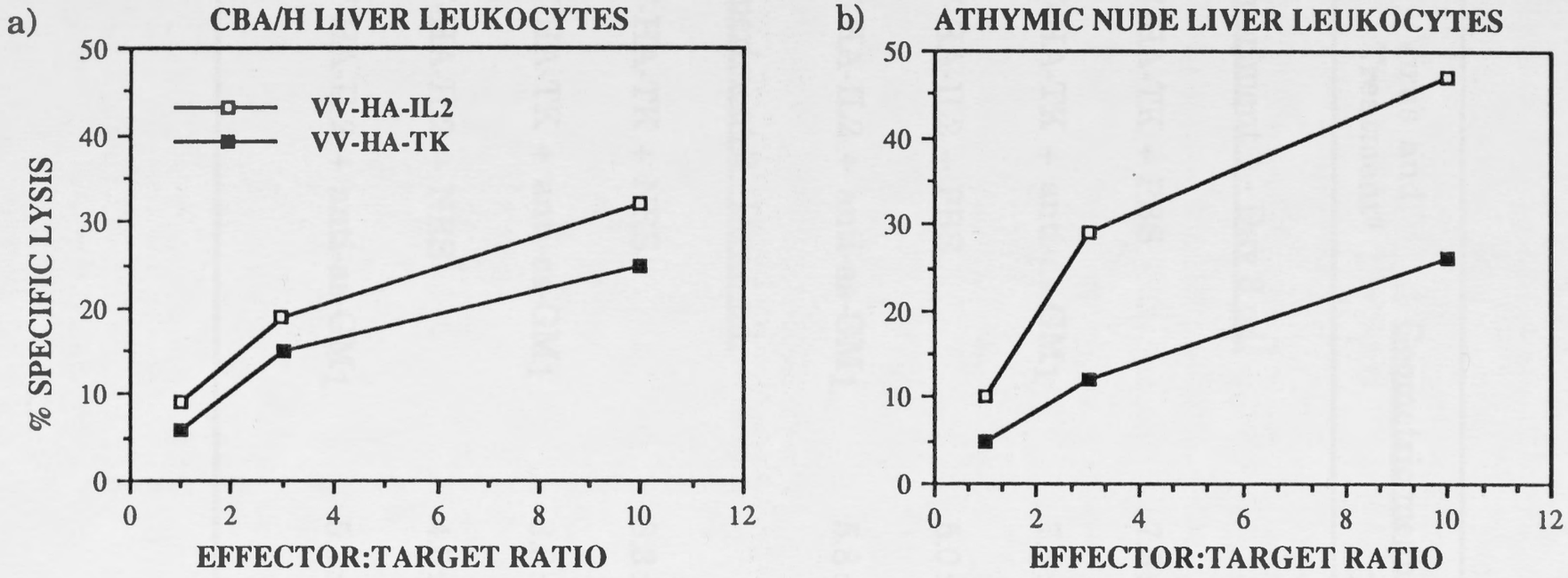


Figure 4. ***Lysis of YAC-1 targets by liver leukocytes from CBA/H mice (a) and athymic nude mice (b).***

Eight week old female CBA/H and athymic nude mice were infected i.v. with 10^7 PFU of VV-HA-TK or VV-HA-IL2 and 3 days later leukocytes were isolated from livers of mice after *in situ* perfusion with collagenase as described earlier.

Table 1.

The effect of as-GM_1 antiserum treatment in nude mice on recombinant VV replication.

Virus and Treatment[a]	Geometric means of log_{10} virus titres ± SEM[b]	
	Ovaries	Lungs
Experiment 1 - Day 3 p.i.		
VV-HA-TK + PBS	7.5 ± 0.3	6.3 ± 0.2
VV-HA-TK + anti-as-GM_1	7.8 ± 0.1	6.7 ± 0.4
VV-HA-IL2 + PBS	5.0 ± 0.2	5.2 ± 0.1
VV-HA-IL2 + anti-as-GM_1	5.8 ± 0.1	6.4 ± 0.4
Experiment 2 - Day 5 p.i.		
VV-HA-TK + NRS	8.3 ± 0.3	6.2 ± 0.2
VV-HA-TK + anti-as-GM_1	8.6 ± 0.1	6.5 ± 0.1
VV-HA-IL2 + NRS	4.3 ± 0.1	4.2 ± 0.1
VV-HA-IL2 + anti-as-GM_1	5.3 ± 0.2	5.8 ± 0.1

Table 1. - continued

[a] Six to 9 week old female outbred nude mice were given 150 µl i.n. antiserum to as-GM_1 (diluted 1:5 in PBS) on days -5, -3, -1, 0, 1 and 2 for Experiment 1 and similarly for Experiment 2 with additional treatments on days 3 and 4. Control mice were given only PBS (Experiment 1) or normal rabbit serum (NRS) (Experiment 2). On day 0, mice were injected i.v. with 10^7 PFU virus, and on the days indicated for each experiment, organs were removed for determination of viral titres.

[b] Data shown are the geometric means of titres of organs from 4 individual mice ± the standard errors of means (SEM).

Table 2.

The effect of as-GM_1 antiserum treatment on the mortality of nude mice after virus infection.

No of mice	Virus and treatment[a]	% mortality	MTD (death on days p.i.)[b]
5	VV-HA-TK + PBS	100	12.0 (9, 12, 12, 13, 14)
4	VV-HA-TK + anti-as-GM_1	100	11.3 (9, 11, 12, 13)
5	VV-HA-IL2 + PBS	nil	-
4	VV-HA-IL2 + anti-as-GM_1	nil	-

[a] Eight week old female outbred nude mice were given 150µl i.v. and 50µl i.n. antiserum to as-GM_1 (diluted 1:5 in PBS) on days -5, -3, -1, 0, 1, 2 and 3. Control mice were given only PBS. On day 0, mice were injected i.v. with 10^7 PFU virus and mortality was recorded. No mortality was observed in mice given antiserum alone (data not shown).

[b] Mean time to death is based on the average of the days individual mice in groups died after infection.

Table 3.

The effect of NK (NK-1.1) cell depletion in normal mice on recombinant VV replication.

Virus and Treatment[a]	Geometric means of log_{10} virus titres± SEM[b] Ovaries	Lungs
C57BL/6J mice (NK-1.1$^+$)		
VV-HA-TK +PBS	7.0 ± 0.2	4.8 ± 0.1
VV-HA-TK + NK-1.1 Mab	7.2 ± 0.3	5.2 ± 0.3
VV-HA-IL2 + PBS	2.9 ± 0.1	3.1 ± 0.3
VV-HA-IL2 + NK-1.1 Mab	5.1 ± 0.1[c]	4.4 ± 0.3[c]
CBA/H mice (NK-1.1$^-$)		
VV-HA-IL2 + PBS	4.4 ± 0.2	4.0 ± 0.1
VV-HA-IL2 + NK-1.1 Mab	4.6 ± 0.1	4.3 ± 0.3

[a] Mice were treated with anti-NK-1.1 Mab as described in the materials and methods section, while controls were similarly given PBS only. Virus titres in organs were determined 3 days after infection with 10^7 PFU.

[b] Data shown are the geometric means of titres of 4 individual organs ± SEM.

[c] significant, $p < 0.01$

Table 4.

The effect of NK (NK-1.1+) cell depletion in normal C57BL/6J mice on Tc cell response and VV-HA-IL2 titres

Infection and/or treatment [a]	E:T	% specific lysis of targets [b] Uninfected macrophages	VV-WR Infected macrophages	EL-4	log_{10}virus titres in ovaries ± SEM[c]
VV-HA-IL2	20:1	5	93	11	< 2.0
	7:1	4	70	4	
	2:1	4	45	2	
VV-HA-IL + NK-1.1 Mab	20:1	4	95	46	
	7:1	3	99	23	4.0 ± 0.5
	2:1	2	74	12	

Table 4. cont.

[a] Six week old female C57BL/6 mice were given 10^7 PFU VV-HA-IL2 i.v. and treated with anti-NK-1.1 Mab as described in Materials and Methods and the assays were carried out six days post-infection.

[b] Peritoneal macrophages from C57BL/6 mice were used as infected or uninfected targets.

[c] Data shown are the geometric means of 4 individual titres for each group.

Table 5.

NK cell responses in normal and beige mice after infection with VV-HA-TK or VV-HA-IL2[a]

Splenocytes	E:T	% specific lysis of YAC-1 targets CBA/H Normal mice	CBA Beige mice
VV-HA-TK-immune	20:1	37	2
	7:1	20	2
	2:1	8	1
VV-HA-IL2-immune	20:1	56	3
	7:1	32	1
	2:1	17	2
Unimmunized controls	20:1	15	2
	7:1	9	2
	2:1	3	0

[a] Splenocyte effectors (pooled from 4 individual spleens) were obtained from 8 week old female normal or beige mice of the CBA background 2 days after i.v. infection with 10^7 PFU virus.

Table 6.

Comparison of Tc cell responses to VV-HA-TK and VV-HA-IL2 in normal and beige CBA mice.[a]

		% specific lysis of L929 targets			
		Uninfected targets		VV-WR Infected targets	
Effectors	E:T[b]	VV-HA-TK -immune	VV-HA-IL2 -immune	VV-HA-TK -immune	VV-HA-IL2 -immune
CBA/H Normal mice	20:1	5	6	65	57
	7:1	2	2	42	35
	2:1	2	0	26	22
Cabg/bg Beige mice	20:1	2	1	70	67
	7:1	1	1	58	52
	2:1	0	0	32	30

[a] Splenocyte effectors (pooled from 4 individual spleens) were obtained from 8 week old female normal or beige mice of the CBA background 6 days after i.v. infection with 10^7 PFU virus.

[b] Effector : target ratio.

Table 7

Enhanced killing of L929 cells infected with VV-HA-IL2 by splenic NK cells.

Effectors [a]	E:T [b]	% specific lysis [c]		
		L929	L929 + VV-HA-IL2	L929 + VV-HA-TK
Normal control	20:1	5	13	9
	7:1	2	7	5
	2:1	0	2	1
VV-HA-IL2 -immune	20:1	18	44	27
	7:1	9	23	17
	2:1	3	11	8

[a] Effector cells were either splenocytes of uninfected CBA/H mice or splenocytes from CBA/H mice infected i.v. with 10^7 PFU of VV-HA-IL2 3 days previously.

[b] Effector : target ratio.

[c] Results shown are representative of 4 separate experiments.

SEM of triplicates were lower than 3% and are omitted for clarity.

Table 8.

Phenotype of liver leukocytes[a] isolated from normal and nude mice infected with VV-HA-IL2 or VV-HA-TK.

Cell surface marker	% positive cells					
	Normal CBA/H mice			Outbred Swiss nude mice		
	uninfected control	VV-HA-IL2 infected	VV-HA-TK infected	uninfected control	VV-HA-IL2 infected	VV-HA-TK infected
Thy-1.2	18	37	25	1.4	8.0	2.6
CD4	9	12	15	0.7	1.1	0.5
CD8	6	5	7	0.5	0.9	1.0
as-GM_1	26	49	34	34.0	58.0	40.0
F4/80	16	21	18	22.0	31.0	34.0

[a] Liver leukocytes were isolated from uninfected controls 3 days after i.v. infection with 10^7 PFU of virus. Isolated leukocytes from 2 mice per group were pooled before staining for flow cytometric analysis. A total of 20,000 fluorescence-activated cell sorting events were analysed for each population and cell surface marker.

Table 9.

Lysis of YAC-1 targets by leucocytes isolated from ovaries of infected mice.[a]

Effectors [a]	E:T [b]	% specific lysis [c]		
		Ficoll-Paque	Percoll	Nylon Wool
Normal control	10:1	2		
	3:1	0	ND[d]	ND
	1:1	0		
VV-HA-TK -immune	10:1	5	4	3
	3:1	3	2	2
	1:1	1	1	2
VV-HA-IL2 -immune	10:1	28	27	11
	3:1	20	17	6
	1:1	15	7	4

[a] Ovarian leucocytes were isolated from normal uninfected female CBA/H mice and CBA/H mice infected i.v. with 10^7 PFU of virus 3 days earlier. Results are representative of 2 separate experiments.

[b] Effector : target ratio.

[c] SEM of triplicates were less than 5%.

[d] Not done.

Table 10.

Phenotype of ovarian effector[a] cells from CBA/H mice infected with VV-HA-IL2 or VV-HA-TK.

Effector cells plus treatment	E:T[b]	% specific lysis					
		YAC-1		L929		L929+VV-WR	
		VV-HA-IL2-immune	VV-HA-TK-immune	VV-HA-IL2-immune	VV-HA-TK-immune	VV-HA-IL2-immune	VV-HA-TK-immune
C	10:1	25	4	21	4	25	4
	3:1	20	1	16	2	21	2
	1:1	12	1	14	1	18	2
C+anti-CD8	10:1	20	5	25	6	31	5
	3:1	19	2	19	3	25	3
	1:1	15	1	16	2	21	4
C+anti-CD4	10:1	25	4	27	5	30	6
	3:1	22	2	20	4	26	5
	1:1	17	0	15	2	22	3
C+anti-as-GM_1	10:1	0	1	10	0	13	1
	3:1	3	1	8	0	11	1
	1:1	0	0	5	0	8	0
C+anti-Thy-1.2	10:1	5	1	17	5	24	6
	3:1	9	1	13	3	20	4
	1:1	2	1	10	2	14	3

[a] CBA/H female mice were infected i.p. with 10^7 PFU virus 3 days before harvesting cells and enriching leucocytes with Ficoll-Paque.

[b] Effector : target ratio.

CHAPTER 5

The role of soluble mediators in viral clearance

INTRODUCTION

The specificity and mechanism of cytotoxicity of antiviral Tc cells have been studied extensively *in vitro*, but their physiological effector function *in vivo* has remained unclear. In this regard, the classical studies by Blanden (1970; 1971a, b) using the mousepox model not only established the importance of Tc cells in mediating recovery from a generalized viral infection but also provided a basis for understanding the mechanisms involved. In response to ectromelia virus infection, the generation of antiviral Tc cells is followed by their migration from the circulation into infectious foci where blood monocytes are recruited through the release of leucotactic factors. The antiviral Tc cells eliminate virus-infected cells by direct or indirect cytolytic mechanisms before progeny virus are produced whereas the recruited monocytes ingest virus or virus-infected cells by virtue of their phagocytic capability. The specifically sensitized Tc may also produce IFN locally which could protect uninfected cells within and around the lesion, increase resistance of monocytes to infection and enhance their virucidal capacity. However, the precise role of soluble mediators such as IFN-γ, produced by antiviral Tc cells, in contributing to antiviral mechanisms is not clearly understood.

Zinkernagel and Althage (1977) suggested two possible mutually exclusive mechanisms which could account for Tc cell-mediated antiviral function *in vivo*. The mechanisms are: (i) Tc cells lyse virus-infected cells before assembly and release of infectious virus progeny; (ii) Tc cells do not lyse infected cells, but release antiviral soluble mediators upon recognition of the relevant viral plus MHC class I antigens. In the same report, evidence was provided in favour of the first mechanism using a mouse-VV-infection model. Virus-immune Tc cells were shown to effectively inhibit replication of VV in acutely infected target cells *in vitro* by destroying infected target cells before assembly and release of progeny virus. These findings, together with the fact that anti-VV Tc cells are demonstrable early in infection (after 3 days), and strong circumstantial evidence for the antiviral activity of Tc from adoptive transfer models *in vivo* led them to suggest that T cells may act cytolytically *in vivo* in some viral infections. Further evidence in support of the first mechanism comes from the work of Lukacher *et al* (1984). They had used murine Tc lymphocyte clones directed to type A influenza virus to examine their *in vivo* antiviral effector activity after adoptive transfer into syngeneic mice

lethally infected with influenza A virus. In a dual-infection model, mice were simultaneously infected with lethal doses of two different subtypes of influenza A virus (H1N1 and H2N2). When cloned Tc cells of H2N2 specificity were transferred to the infected mice, the pulmonary viral titres of the strain recognized by the Tc cell clone only was reduced. The high level of specificity exhibited by Tc cells led these investigators to conclude that Tc cells mediate antiviral protective effects *in vivo* by direct cytolysis of virus-infected cells in an antigen-specific manner and not by non-specific host defense mechanisms such as the release of soluble factors.

The above studies seem adequately convincing, but they fail to accommodate the possibility that both cytolysis of infected cells and release of antiviral factors are not mutually exclusive. Furthermore, such soluble factors produced by T cells are secreted locally into a limited space between interacting cell types (Poo *et al.*, 1987).

Based on their findings that clones of murine antigen-specific, H-2-restricted, Tc cells released high levels of IFN-γ when co-cultured with allogeneic cells presenting antigen, Klein and others (1982) suggested that Tc cells, on binding to antigen complexed with class I MHC, secreted IFN-γ in addition to cell lysis. Tc cells that recognized and killed virus-infected cells would help prevent spread of infection if in addition they released IFN-γ at the local site of infection. IFN-γ–induced upregulation of class I MHC expression could enhance recognition of virally infected target cells by Tc cells (Bukowski and Welsh, 1985; Schiltknecht and Ada, 1985c) and, furthermore, IFN-γ may activate macrophages and enhance their ability to destroy virus (Blanden, 1971b). The demonstration by Morris *et al* (1982) that an anti-influenza Tc cell clone that conferred protection against lethal influenza infection in mice also produced IFN-γ when exposed to histocompatible, virus-infected targets *in vitro* led them to speculate that both direct cytolysis of virus-infected cells and secretion of IFN-γ may be important in the control of an infection.

The mechanism by which NK cells mediate antiviral function *in vivo* is still not entirely known. Moreover, the recognition element(s) for NK cells have not been characterised although a recent report (Müllbacher and King, 1989) indicated that MHC class 1-unassociated β2-m may serve as a ligand for NK cell-mediated lysis of targets.

The murine cytomegalovirus (MCMV) infection of the mouse is presently the most convincing virus system in which NK cells have been demonstrated to contribute to antiviral mechanisms (reviewed in Welsh, 1986). Evidence that cytolytic NK cells may be important in control of MCMV infection is essentially based on the findings that mice with genetically high levels of NK activity are more resistant than those with genetically low levels (Bancroft *et al.*, 1981). Mice bearing the beige mutation (Roder and Duwe, 1979), and therefore deficient in cytolytic NK cells, are also highly susceptible (Shellam *et al.*, 1981). Furthermore, an NK-cell like clone with *in vitro* cytolytic activity protected baby mice from MCMV infection (Bukowski *et al.*, 1985) but the *in vivo* protective effect was lost when the clone failed to lyse target cells *in vitro* after long term culture (quoted in Welsh, 1986). Whether NK cells contribute to antiviral mechanisms *in vivo* by cytolytic activity and/or secretion of antiviral factors is still unclear.

NK cells may secrete all 3 species of IFNs. However, the type of IFN produced appears to depend on the stimulus. For example, cloned murine NK cells produce IFN-α or -β on stimulation with Sendai virus (Handa *et al.*, 1983) but otherwise produce IFN-γ following stimulation with IL-2 (Handa *et al.*, 1983; Young and Ortaldo, 1987). In at least two independent reports on the evaluation of toxicity and therapeutic efficacy of high dose IL-2 using mouse models, the toxic effects were in part attributed to the preferential secretion of IFN-γ and/or TNF-α by IL-2-activated NK cells (Gately *et al.*, 1988; Peace and Cheever, 1989). These reports have 2 important implications. Firstly, they indicate that IL-2 can trigger NK cells to produce cytokines with known antiviral and antitumour activities. Secondly, while IL-2-activated NK cells can play an important role in orchestrating beneficial effects such as control of viral infection (Weinberg *et al.*, 1986; Bukowski *et al.*, 1988) and antimetastatic activity (Hinuma *et al.*, 1987), they can also induce toxic manifestations. The undesirable effects of NK cell-mediated toxicity appear to be related to the high dose of IL-2 used (Cotran *et al.*, 1987).

The fact that resting NK cells, but not T cells, respond to IL-2 and produce IFN-γ is most probably due to the constitutive expression of the p75 subunit of the IL-2R on NK cells (Siegel *et al.*, 1987; Tsudo *et al.*, 1987). Resting T cells do not express the p55 subunit of the IL-2R. Although

some T cells do express the p75 subunit of the IL-2R, they require antigenic or other mitogenic stimuli to respond to IL-2. These observations may perhaps explain why NK cells are more rapidly activated in mice infected with VV-HA-IL2 compared to VV-HA-TK.

Evidence presented in the preceding chapter from studies using nude, beige and normal mice infected with VV-HA-IL2 suggested both NK cell-dependent and -independent mechanisms contributed to viral clearance and survival of nude mice. Control of the virus infection by NK cells could either be mediated by direct cytolysis of infected cells and/or through the release of antiviral factors which directly or indirectly prevent virus spread. The release of IFN-γ or cytokines that activate macrophages and, hence, increase their virucidal capacity could be involved in this process (Blanden, 1971b; Blanden, 1982; Blanden *et al.*, 1976). It is also likely that expression of IL-2 during infection with VV-HA-IL2 induced other leukocytes (non-NK cells) to produce potentially effective concentrations of antiviral factors such as IFN-γ or TNF-α. These cytokines are known to synergise with IFN-α and/or -β (Wong *et al.*, 1986), both of which are normally produced during virus infections, to exert potent antiviral activity.

Therefore, the roles of host-derived cytokines such as IFN-γ, TNF-α and IFN-β in the recovery from VV-HA-IL2 infection was investigated. The approach taken to evaluate the relative importance of these soluble mediators was by the use of specific Mab/antisera to inhibit their activity *in vivo* during infection with VV-HA-IL2. Viral titres, morbidity and mortality were assessed in animals treated with these antibodies. In addition, the role of mononuclear phagocytes in the process of recovery of nude mice from VV-HA-IL2 infection was investigated.

MATERIALS AND METHODS

Mice.

As described in Chapter 2

Viruses.

As described in Chapter 2

Antisera/Mab

Purified rat anti-mouse IFN-γ, a neutralizing Mab (5,700 U/mg) was obtained from Lee Biomolecular Research Inc., San Diego, CA. One U of this antibody neutralised 10U of the antiviral activity of murine IFN-γ *in vitro*.

A polyclonal anti-human TNF-α antibody (HT-2 4/14/88), originally raised in rabbits by Drs. S. Kunkel and D. Remick, was kindly provided by Dr. R. Marks, University of Michigan Medical School, Ann Arbor, Michigan. This antibody neutralised the biological activity of murine TNF-α *in vitro* and *in vivo*.

Rabbit antiserum to mouse IFN-β (neutralizing antibody with activity of 10,000 unit/ml) was obtained from Lee Biomolecular Research Inc., San Diego, CA.

Hybridoma cell lines, clone GK1.5 (anti-CD4) (Dialynas *et al.*, 1983b) and clone 49.11.1 (anti-CD8/Ly2.1) (Hogarth *et al.*, 1982) were kindly provided by Dr. R. Ceredig (John Curtin School of Medical Research). These antibodies were obtained as ascites, grown in pristane-primed outbred nude mice and used after partial purification as described in Chapter 2.

***In vivo* cell depletion with Mabs.**

Normal CBA/H mice were depleted of $CD4^+$ or $CD8^+$ T cells using the appropriate Mabs described above. Mice were given i.p. 500 µl of anti-CD4 or 500 µl of anti-CD8 Mabs 3 times on alternate days prior to infection (days -5, -3, -1), on the day of infection (day 0), and for 2 consecutive days after infection (days 1 and 2). This procedure is based on titrations for the depletion of T cells, perfomed with either Mab. Efficiency of depletion was assessed by flow cytometry as described in Chapter 4.

Treatment with antisera/Mab to cytokines.

The rat-anti-mouse IFN-α Mab was diluted in PBS and a 200 µl volume was given i.p. (varying concentrations, depending on the experiment) to nude or normal mice on the days indicated in the Results section. Generally, mice were given the Mab one day prior to infection, on the day of infection and thereafter everyday except for the day of sacrifice. In experiments where mortality in nude mice was assessed, the Mab was given one day prior to infection, on the day of infection and for 3 consecutive days after infection.

A 100 µl volume of the anti-human TNF-α antibody, sufficient to neutralize the *in vivo* biological activity of murine TNF (Dr. R. Marks, personal communication) was administered i.v. to nude mice one day prior to infection, on the day of infection and thereafter for 3 consecutive days p.i..

The rabbit antiserum to mouse IFN-β was given i.p. to mice as follows: 500U on days 0 (day of infection), 1 and 2, and 400U on days 3 and 4 post-infection.

Recombinant IL-2 and IFN-γ.

Affinity column purified recombinant (r) murine IL-2, with a specific activity of 64,000U/ml, was kindly provided by Dr. P. Hodgkin, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA., USA. Recombinant murine IFN-γ (300,000U/ml) was a kind gift of Boehringer Ingelheim, Vienna, Austria.

Nude mice that had been infected 1 h earlier with VV-HA-TK were given i.p. 600U of recombinant murine rIL-2 or rIFN-γ and thereafter every 8 h for a period of 5 days. Uninfected controls were treated similarly to assess the toxicity of the lymphokines.

Macrophage depletion *in vivo* with silica.

Silica (silicon dioxide), particle size between 0.5 to 10 μ (more than 80% between 1 and 5 μ) was obtained from the Sigma Chemical Company, St Louis, MO, USA. Mice were depleted of macrophages with silica based on methods reported elsewhere (Allison, 1976).

Nude mice were given silica (suspended in PBS and sonicated at 50 watts for 3x5 second) i.p. (5 mg) and i.v. (5 mg) on days -1, 3, 6 and 9. Treatment via the two routes is effective for the depletion of blood monocytes, peritoneal and organ associated macrophages (Allison, 1976).

Assay for IL-2.

The presence of IL-2 in sera, peritoneal fluids or ascites of mice was assessed by CTL-L cell proliferation and uptake of ^{3}H-thymidine as described in Chapter 2.

Virus titration.

As described in Chapter 2.

Cytotoxic assays.

As described in Chapter 3.

Assay for IFN-γ in nude mouse splenocyte culture supernatants.

Splenocytes from outbred nude mice were cultured in the presence of rIL-2 to determine whether as-GM_1^+ and as-GM_1^- cells produced IFN-γ. Whole splenocytes ($5x10^6$ cells treated with C only) were cultured in 1ml of complete EMEM containing $5x10^4$ M 2-mercaptoethanol and 100U rIL-2 for 3 days at 37°C in a humidified atmosphere containing 5% CO_2 in air. Similarly, splenocytes depleted of as-GM_1^+ ($5x10^6$ cells treated with anti-as-GM_1 + C) were cultured under the same conditions and controls were not exposed to IL-2. Presence of IFN-γ in the culture supernatants was determined using a commercial sandwich ELISA (Holland Biotechnology bv, Leiden, Netherlands). The ELISA was performed according to the manufacturers' instructions. IFN-γ activity (U/ml) was calculated from optical density values using a standard curve.

RESULTS

Enhanced VV-HA-IL2 replication *in vivo* after treatment with IFN-γ Mab in nude mice.

Nude mice infected i.v. with 10^7 PFU VV-HA-TK or VV-HA-IL2 were treated with a neutralising Mab to IFN-γ and virus titres were determined in organs 3 and 5 days after infection.

Three days after infection, VV-HA-TK titres in ovaries and lungs of anti-IFN-γ Mab-treated mice were 0.8 $\log_{10}$ PFU and 0.9 $\log_{10}$ PFU higher than in PBS-treated controls respectively (Table 1). On the other hand, Mab treatment resulted in increases of 2.5 $\log_{10}$ PFU in ovaries and 1.8 $\log_{10}$ PFU in lungs of VV-HA-IL2-infected mice.

Five days after infection, treatment with the Mab caused 0.4 $\log_{10}$ increase in VV-HA-TK titres in ovaries and lungs compared to controls given PBS (Table 1). In contrast, VV-HA-IL2 titres were higher by 4.8 $\log_{10}$ in ovaries and 2.3 $\log_{10}$ in lungs of nude mice that had received the Mab to IFN-γ. Furthermore, titres of VV-HA-IL-2 in Mab-treated mice were now comparable to thè titres reached by VV-HA-TK.

These data indicate that in nude mice early during an infection with VV-HA-TK, IFN-γ exerted antiviral function but the effect was lower on day 5 than on day 3. During infection with VV-HA-IL-2, treatment with anti-IFN-γ Mab resulted in greater enhancement viral replication than with VV-HA-TK, suggesting that virus-encoded IL-2 may have induced the secretion of IFN-γ.

The ability of nude mice to survive infection with VV-HA-IL2 is inhibited by anti-IFN-γ Mab.

The above data suggested that IFN-γ induced by virus-encoded IL-2 played an important role in virus clearance. Experiments were therefore undertaken to assess the effect of anti-IFN-γ Mab treatment on the survival of nude mice infected with VV-HA-IL2.

The results of a representative experiment are shown in Table 2. Treatment with 500U of anti-IFN-γ Mab in VV-HA-TK-infected mice did not significantly ($p > 0.05$) affect the mean time to death (MTD) of about 15 days compared to mice given VV-HA-TK only. However, nude mice infected with VV-HA-IL2 and treated with the antibody died from the fifth day onwards after infection, with a MTD of 9.6 days. This is in sharp contrast to the group of mice given VV-HA-IL2 only, where no mortality was recorded. The MTD was dependent upon the dose of anti-IFN-γ Mab administered. Mice infected with VV-HA-IL2 and treated with several doses of 100U Mab had a MTD of 11 days whereas treatment with 500U Mab resulted in a MTD of 6 days (Table 3).

IL-2-mediated toxicity in mice infected with VV-HA-IL2 and treated with anti-IFN-γ Mab.

Some nude mice that had been infected with VV-HA-IL2 and treated with anti-IFN-γ Mab died very rapidly, between days 5 to 8 p.i., with signs of erythematous eruptions all over the body and no distinct pox lesions. Mice that died after 9 to 10 days p.i. had obvious signs of vaccinial disease with pox lesions on tail and feet but with no erythematous eruptions. It is likely that the enhanced replication of VV-HA-IL2 after treatment with IFN-γ Mab, resulted in the production of toxic levels of IL-2.

Apart from exhibiting erythroderma, nude mice that appeared to suffer from the toxic effects of IL-2 had ascites and massive accumulation of mononuclear cells in the peritoneal and pleural cavities. These pathological findings are consistent with IL-2 toxicity which may be manifested in the form of vascular leak syndrome (Cotran *et al.*, 1987). Evidence for vascular leakage was also sought by histologic examination of skin sections obtained from VV-HA-IL2 infected, antibody-treated mice. There was mild papillary oedema, some perivascular mononuclear cell infiltration and red blood cell extravasation in the dermal interstitial

spaces (Fig. 1a). There was occasional engorgement of capillaries with red blood cells. These findings suggested increased permeability of the capillary walls. Similar pathology was not seen in histological sections of skin from mice that had been infected with VV-HA-IL2 only (Fig. 1b). Massive mononuclear cell infiltration was also noted in liver sections, with large areas of necrosis, in mice infected with VV-HA-IL2 and treated with the Mab (Fig. 1c), but not in livers of mice that had been infected with VV-HA-TK and treated with Mab (Fig. 1d).

Varying levels of IL-2 were demonstrable in sera, ascites and peritoneal fluids of mice that displayed the pathology described above (data not shown). No IL-2 was detected in sera or peritoneal fluids from mice that had been infected with VV-HA-IL2 only or mice infected with VV-HA-TK and treated with anti-IFN-γ Mab. There was a 5-10-fold increase in the number of peritoneal cells in mice with obvious pathology . About 1-2x10^6 mononuclear cells were obtained from the peritoneal cavity of mice that had been infected with VV-HA-IL2. However, in mice that were infected with VV-HA-IL2 and treated with anti-IFN-γ Mab, up to 10^7 peritoneal cells were recovered. These cells had potent cytolytic activity on YAC-1 targets which was more than 9-fold higher than activity induced by infection with VV-HA-IL2 only (Table 4). They were as-GM$^{\pm}$ and Thy-1.2$^{\pm}$. Although treatment with anti-as-GM_1 and C reduced the cytolytic activity on YAC-1 targets by more than 9-fold, substantial lytic activity still remained (Table 4).

Effect of treatment with anti-IFN-γ Mab of VV-HA-IL2-infected nude mice on splenic NK cell activity.

Investigations were carried out to determine if treatment with the anti-IFN-γ Mab in VV-HA-IL2-infected nude mice inhibited NK cell activation, thereby preventing NK cell-mediated antiviral function *in vivo*. Five days after i.v infection with 10^7 PFU of VV-HA-TK, levels of NK activity (lysis of YAC-1 targets) in Mab-treated mice were slightly lower than untreated controls given virus only (Fig. 2). In contrast, the splenic NK activity in mice that had been infected i.v. with 10^7 PFU of VV-HA-IL2 and treated with the Mab was more than 3-fold higher than activity induced by infection with VV-HA-IL2 alone (Fig. 2). These data indicate that

treatment with the anti-IFN-γ Mab in VV-HA-IL2-infected mice did not prevent NK cell activation.

Splenocytes from VV-HA-TK-infected mice mediating lysis of YAC-1 targets were as-GM_1^+, Thy-1.2±, $CD4^-$ and $CD8^-$. Lysis of YAC-1 targets by splenocytes from VV-HA-IL2-infected mice was mainly due to as-GM_1^+, Thy-1.2±, $CD4^-$ and $CD8^-$ effectors and to a lesser extent by effectors that were as as-GM_1^-, Thy-1.2±, $CD4^-$ and $CD8^-$ (data not shown). Splenocytes obtained from VV-HA-IL2-infected mice that had been treated with anti-IFN-γ Mab were as-GM_1±, Thy-1.2±, $CD4^-$ and $CD8^-$ and were not completely lysed by treatment with anti-as-GM_1 and C (data not shown) as was noted for the peritoneal cells (Table 4).

Effect of administration of rIL-2 or rIFN-γ on mortality and survival time of nude mice infected with VV-HA-TK.

The above data clearly established that in VV-HA-IL2-infected mice IFN-γ is involved in the rapid clearance of virus and recovery of nude mice. Therefore it was of interest to determine whether administration of exogenous rIL-2 or rIFN-γ during infection with VV-HA-TK contributed to recovery of nude mice.

Groups of 3 - 5 outbred nude mice infected i.v. with 10^7 PFU VV-HA-TK were given 600U of rIL-2 or rIFN-γ i.p. every 8 h for a period of 5 days. The rationale for this protocol was based on the kinetics of VV-HA-IL2 clearance reported in Chapter 2. Morbidity and mortality in these and other groups given virus alone or the recombinant cytokines alone were assessed.

No morbidity or mortality was recorded in groups of mice given only rIL-2 or rIFN-γ. All mice given VV-HA-IL2 alone survived with no overt disease. Nude mice infected with VV-HA-TK alone showed signs of disease by 6 days p.i. and all mice died with a MTD of 12.2 days (Table 5). Treatment with exogenous rIL-2 or rIFN-γ delayed the onset of disease signs which appeared between 11-16 days p.i., and significantly ($p < 0.001$) prolonged survival of nude mice. Nevertheless, all mice that had been infected with VV-HA-TK and treated with either rIL-2 or rIFN-γ succumbed to disseminated disease and died with MTD of 23.4 and 25.8 days, respectively.

TNF-α or IFN-β may not be crucial for the recovery of nude mice from VV-HA-IL2 infection.

While the data presented above convincingly indicate that IFN-γ is an important component in the recovery of nude mice from an infection with VV-HA-IL2, it is possible that other soluble factors could have contributed to antiviral mechanism(s), either on their own or in synergy with IFN-γ. In this regard, the most likely known participants are TNF-α, which can be triggered by IL-2 or IFN-γ, and IFN-α and IFN-β which are normally produced during a virus infection. Since the antisera to TNF-α and IFN-β were only available in limited amounts, preliminary experiments were carried out to determine if these reagents inhibited the recovery of nude mice from an infection with VV-HA-IL2. Groups of 4 nude mice treated with anti-TNF-α or anti-IFN-β were infected i.v. with 10^7 PFU VV-HA-IL2 and were observed for morbidity and mortality. Although some morbidity was recorded in the group that had been treated with anti-IFN-β antibody, all mice that had been infected with VV-HA-IL2 and treated with anti-TNF-α or anti-IFN-β recovered from the infection. No virus persisted in organs of these mice at 20 days p.i. While these studies are not backed up by data on virus titres in organs early in the infection, they nevertheless suggest that TNF-α or IFN-β may not be crucial in mediating the recovery of nude mice.

The role of IFN-α was not investigated.

The role of mononuclear phagocytes in the recovery mechanism(s).

Monocytes and macrophages have a well defined antiviral role *in vivo* Since IFN-γ has been shown to be crucial for the recovery of nude mice from an infection with VV-HA-IL2, it is possible that IFN-γ-mediated antiviral mechanism(s) were in part due to activated monocytes/macrophages. IFN-γ is a potent monocyte/macrophage activating factor and anti-IFN-γ Mab can abrogate the antiviral and antitumour activities of these mononuclear phagocytes (Spitalny and Havell, 1984). To investigate the possible involvement of this class of cells, nude mice were treated with silica, a procedure known to deplete or inactivate macrophages (Allison, 1976). Groups of 5 nude mice that had been treated with silica were infected i.v. with 10^7 PFU VV-HA-TK or VV-HA-IL2. Controls were treated with silica only or infected with viruses only. Morbidity and mortality were assessed.

Treatment with silica, did not acccelerate the MTD of nude mice that had been infected with VV-HA-TK (Table 6). Two of the 5 mice that had been infected with VV-HA-IL2 and treated with silica died 30 days after infection with obvious signs of a disseminated vaccinial disease. The remaining 3 mice in the group were sacrificed 32 days after infection in order to determine if virus had been cleared. About 3.8 $\log_{10}$ PFU virus was recovered from the lungs and 4.4 $\log_{10}$ PFU was recovered from the brains of these mice (Table 6). Treatment with silica also resulted in lower splenic NK cell activity in uninfected or virus-infected mice (data not shown).

Both as-GM_1^+ and as-GM_1^- nude mouse splenocytes produce IFN-γ *in vitro*.

In the previous Chapter, depletion of as-GM_1^+ (NK) cells in nude mice was shown to result in enhanced VV-HA-IL2 replication (Chapter 4, Table 1), plus signs of morbidity but no mortality. As discussed in Chapter 4, a possible explanation for the failure of NK cell-depleted nude mice to die from VV-HA-IL2 infection was that both as-GM_1^+ (NK) and as-GM_1^- (NK precusors and non-NK) cells may have contributed to virus clearance, either by cytolysis of virus-infected cells or secretion of antiviral factors. It was therefore of interest to determine if as-GM_1^+ and as-GM_1^- splenocytes from nude mice produced IFN-γ on stimulation with IL-2. Nude mouse splenocytes, either whole or depleted of as-GM_1^+ cells, were cultured in media containing 100U/ml rIL-2 for 3 days following which the presence of IFN-γ in cultures was assayed using an ELISA. More than 40U/ml of IFN-γ was detected in whole splenocyte culture supernatants, whereas between 26-30U/ml of the lymphokine was detected in cultures depleted of as-GM_1^+ cells. No IFN-γ was detected in control cultures that had been maintained in the absence of IL-2.

The role of IFN-γ in the clearance of virus in normal mice.

Based on the data obtained in studies with nude mice, it was likely that IFN-γ, induced by virus-encoded IL-2, mediated the rapid clearance of VV-HA-IL2 in normal mice. Therefore experiments were carried out using normal CBA/H mice to assess the role of IFN-γ in rapid viral clearance.

Groups of female CBA/H mice were treated with the IFN-γ Mab one day prior to infection (day -1), on the day of infection (day 0) and for 2 consecutive days after infection. Three days after i.v. infection with 10^7 PFU virus, mice were sacrificed to determine viral titres in organs and splenic NK activity.

The antibody treatment resulted in enhanced VV-HA-TK and VV-HA-IL2 replication in ovaries and lungs. Mean titres of VV-HA-TK increased significantly ($p < 0.01$) by 0.9 $\log_{10}$ PFU in ovaries and 1 $\log_{10}$ PFU in lungs of antibody-treated animals (Table 7). The effect of IFN-γ Mab treatment was more marked in the case of VV-HA-IL2 replication. The mean VV-HA-IL2 titre increased by 3.7 $\log_{10}$ in ovaries and 1.5 $\log_{10}$ in lungs compared to titres in control mice (Table 7).

In normal mice infected with VV-HA-TK or VV-HA-IL2, treatment with anti-IFN-γ antibody resulted in enhanced NK cell activity, but the effect was more marked in VV-HA-IL2-infected mice (Table 7).

Six days after infection, the Tc cell responses to VV-HA-TK and VV-HA-IL2 were elevated in mice treated with anti-IFN-γ Mab and were at least 3-fold higher than the responses in untreated animals (Table 8). Although by this time VV-HA-IL2 was undetectable in ovaries of control mice, in anti-IFN-γ Mab-treated mice, up to 5.6 $\log_{10}$ PFU VV-HA-IL2 was recovered (Table 8). The mean VV-HA-TK titre also increased from 6.5 $\log_{10}$ PFU in the control group to 7.5 $\log_{10}$ in the Mab-treated mice (Table 8). No virus was recovered from lungs at 6 days p.i.

The above data clearly indicate that treatment with anti-IFN-γ Mab in normal mice did not inhibit NK cell activation or the induction of antiviral Tc cell response. However, IFN-γ, presumably secreted by NK and T cells, appears to be an important factor in viral clearance, especially in the case of VV-HA-IL2.

The role of $CD4^+$ and $CD8^+$ T cells in rapid VV-HA-IL2 clearance by possible IFN-γ secretion.

In normal mice, both T cells and NK cells are known to produce IFN-γ. In the previous Chapter, it was demonstrated that depletion of NK cells in normal mice led to enhanced VV-HA-IL2 replication. Based on the data presented in Chapter 4 and in this Chapter, there is sufficient evidence to

suggest the NK cells could have contributed to antiviral mechanism(s) *in vivo* by cytolysis of virus infected cells and/or secretion of antiviral factors like IFN-γ. Both $CD4^+$ and $CD8^+$ T cells were not represented in the cytolytic effector populations evident up to day 3 after infection (see Chapter 3, Tables 2 and 4). It was of interest, however, to determine if these two populations of T cells contributed to antiviral mechanisms *in vivo*, during the first 3 days after infection through secretion of IFN-γ. Therefore normal CBA/H mice were depleted of $CD4^+$ or $CD8^+$ T cells by repeated injections with anti-CD4 and anti-CD8 Mab, infected with 10^7 PFU of VV-HA-IL2 i.v. and 3 days later, the viral titres in organs were determined.

While the depletion of $CD8^+$ T cells had virtually no effect ($p > 0.05$) on viral titres, the mean VV-HA-IL2 titres in lungs and ovaries of anti-CD4 antibody treated mice were significantly ($p < 0.001$) higher than control mice (Table 9).

DISCUSSION

The currently held view with regard to the role of Tc cells in host defense to viral infection is based on the capacity of these effectors to recognize viral peptide in association with the MHC Class I antigen on the surface of infected cells followed by cytolysis (Zinkernagel and Doherty, 1974; Zinkernagel and Althage, 1977; reviewed in Zinkernagel and Doherty, 1979; Lukacher *et al.*, 1984; Townsend *et al.*, 1986a) and possibly, local release of IFN-γ (Blanden, 1971a; Klein *et al.*, 1982; Morris *et al.* 1982). The IFN-γ, released in an antigen-specific and H-2-restricted manner, could control further spread of virus by a variety of mechanisms, but its relative importance is unclear. On the other hand, while NK cells have been implicated to mediate antiviral function *in vivo* (Bukowski *et al.*, 1984; reviewed in Welsh, 1986), the the relative importance of NK cell-mediated cytolytic activity versus NK cell-secreted antiviral factors is not known.

The results presented in this Chapter demonstrate very clearly that IFN-γ played a crucial role in the efficient clearance of VV-HA-IL2 and the recovery of nude mice from an infection with this virus. The IL-2-induced IFN-γ was also involved in efficient clearance of VV-HA-IL2, and to a lesser extent VV-HA-TK, in normal mice. IL-2 induces and regulates the production of IFN-γ by T lymphocytes and NK cells (Farrar *et al.*, 1981; Torres *et al.*, 1982; Handa *et al.*, 1983; Trinchieri *et al.*, 1984; Young and Ortaldo, 1987). Since nude mice lack functional T cells which are the principal producers of IL-2, the virus-encoded IL-2 was essential for nude mouse lymphoid cell activation and IFN-γ secretion. Although lymphoid cells from aged (4-6 month old) N:NIH(S) II nude mice were previously shown to be capable of secreting IL-2 in response to ConA (MacDonald *et al.*, 1982), a quantitative analysis of the precursors of IL-2 producing cells in that system showed a 5-to-10-fold deficiency in precursor frequency among nude lymphoid cells (MacDonald and Lees, 1984). Therefore, if IL-2 is crucial for the induction and regulation of IFN-γ production, then nude mouse lymphoid cells would be expected to produce only low levels of IFN-γ which may be insufficient for the control of a virus infection. In normal mice, optimal levels of IL-2 production by T lymphocytes would ensure the induction and regulation of relevant concentrations of IFN-γ secretion.

As-GM_1^+ NK cells are thought to be the major source of IFN-γ production in T-cell deficient mice (Bancroft *et al.*, 1989). The present data suggests, however, that as-GM_1^- cells, under appropriate conditions, may secrete IFN-γ. Both as-GM_1^+ and as-GM_1^- nude mouse splenocytes were shown earlier (Chapter 3, Tables 7 and 8) to cause lysis of YAC-1 targets. However, there is no evidence on the question of whether these cytolytic populations were also responsible for IFN-γ secretion *in vivo*. It is also not clear if the as-GM_1^- nude mouse lymphoid cells are progenitors of NK cells or non-NK cells.

Since both T cells and NK cells can produce IFN-γ in normal mice, it is likely that both these populations contributed to IFN-γ secretion after infection with VV-HA-IL2. Data presented in this and the preceding Chapters, from experiments in which Mabs were used to deplete specific cell subsets in normal mice, indicated that NK and $CD4^+$ T cells may have contributed to inhibition of VV-HA-IL2 replication by the secretion of IFN-γ. However, the enhanced VV-HA-IL2 replication in normal mice depleted of $CD4^+$ T cells is by no means proof that this subset of T cells could have contributed to antiviral function *in vivo* only by secretion of IFN-γ. The finding that $CD8^+$ T cell depletion did not affect VV-HA-IL2 titres is consistent with data presented earlier (Chapter 3, Table 7), which indicated that $CD8^+$ T cells did not play a role in viral clearance during the first 3 days after infection.

The anti-IFN-γ Mab did not inhibit NK cell activation or Tc cell generation, indicating the possiblity that the antiviral function of these effector cells could have been impaired *in vivo* and also concurring with the report by Sayers and others (1986) that the potentiating effect of IL-2 on the cytolytic activity of NK cells is not mediated by IFN-γ. In fact, the enhancement of NK cell cytotoxicity mediated by IL-2 is known to precede by several hours the appearance of detectable IFN-γ in culture supernatants (Sverdersky *et al.*, 1984; Trinchieri *et al.*, 1984).

The finding that a proportion of VV-HA-IL2-infected nude mice that had been treated with anti-IFN-γ Mab died from IL-2 toxicity is consistent with increased IL-2 production due to enhanced VV-HA-IL2 replication. This is further evidence that IL-2 is produced *in vivo* during infection with VV-HA-IL2. IL-2 was not detected in biological fluids of mice infected with VV-HA-TK, whether or not they had been treated with anti-IFN-γ Mab.

Although IL-2 could not be demonstrated in sera of mice infected with VV-HA-IL2 but untreated with ant-IFN-γ Mab, this may reflect the fact that IL-2 is produced locally at sites of infection, at levels that are sufficent to mediate a cascade of immune mechanisms but not high enough to be detected systemically. In this regard, administration of exogenous rIL-2 or rIFN-γ did not allow recovery but prolonged survival of nude mice infected with VV-HA-TK. Because lymphokines are usually produced as paracrine (and autocrine) factors secreted into a limited space between interacting cell types (Poo *et al.*, 1987), virus-encoded IL-2 produced locally would be expected to operate (in inducing antiviral immune mechanisms) more effectively and efficiently than the systemic administration of lymphokines which also have very short half lives *in vivo*. The data may reflect the fact that it is not possible to mimic, by administration of exogenous rIL-2 or rIFN-γ, conditions under which VV-encoded IL-2 or the IL-2-induced IFN-γ operate *in vivo*. It may also underscore the importance of local immune mechanisms which operate at the sites of virus infection.

IL-2 in foci of infection may have induced the secretion of other cytokines which are believed to mediate, at least in part, the toxic effects of IL-2 (Cotran *et al.*, 1987; Gately *et al.*, 1988, Peace and Cheever, 1989). The presence of one such cytokine, TNF-α, could not be demonstrated in the biological fluids of mice that were suffering from IL-2 toxicity.

IFN-γ could have contributed in a number of ways to limit viral replication *in vivo*. First, it is possible that IFN-γ may have had a direct antiviral effect on cells either through limiting viral replication or preventing infection of other cells. Recent evidence (Leist *et al.*, 1989; Kohonen-Corish *et al.*, 1990; Ruby and Ramshaw, submitted) is consistent with an antiviral role for IFN-γ *in vivo*.

Second, IFN-γ, could have mediated antiviral funciton *in vivo* through monocyte/macrophage activation and data presented earlier is consistent with a role for these mononuclear phagocytes. While the complete depletion of monocytes/macrophages is unlikely to be achieved *in vivo*, the data provide sufficient evidence which supports the earlier suggestion that monocytes/macrophages (most likely activated by IFN-γ) may have contributed, in part, to the IL-2-induced antiviral mechanisms which enabled nude mice to recover from infection with VV-HA-IL2. Nude mice

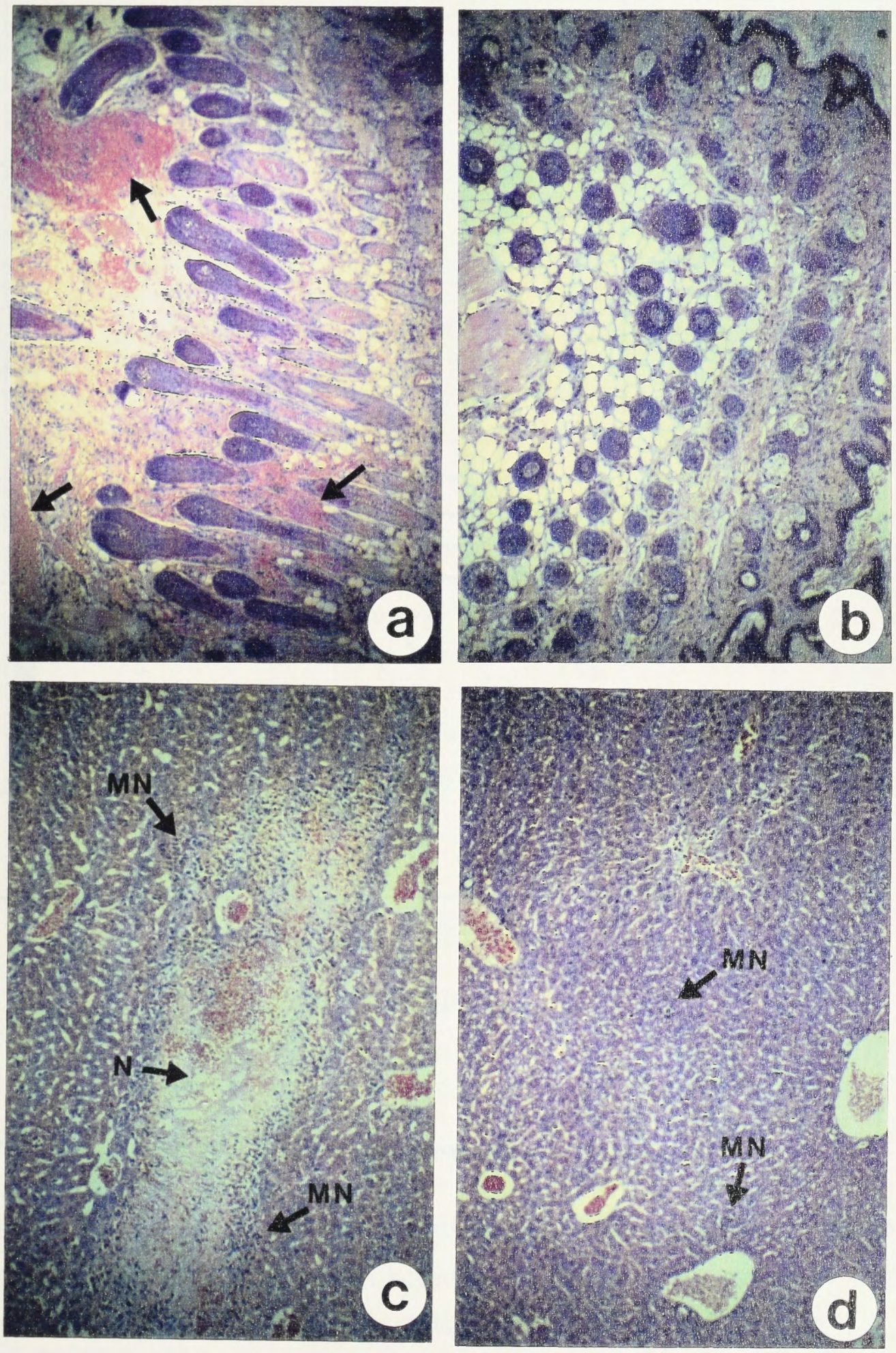
treated with silica were unable to clear VV-HA-IL2. Therefore, mononuclear phagocytes seem essential for the full expression of IFN-γ mediated antiviral function. The finding that silica treatment reduced NK activity supports the possibility that persistence of VV-HA-IL2 in silica-treated mice could have been partly due to impaired NK cell function *in vivo*.

The third mechanism by which IFN-γ could have contributed to antiviral function *in vivo* is based on the ability of this lymphokine to regulate the induction or upregulation of the expression of class I and class II MHC gene products and β_2-microglobulin (β_2-m). The membrane-bound class I MHC glycoproteins serve as recognition elements for $CD8^+$ Tc lymphocytes during immune elimination of virus-infected cells. Recently, class I MHC-unassociated β_2-m has been implicated as a ligand for NK cell recognition and lysis of target cells (Müllbacher and King, 1989). Treatment with a neutralizing Mab to IFN-γ *in vivo* could have interfered with the induction or upregulation of both the T- and NK-cell ligands, thus affecting the recognition, and triggering of lysis of virus infected cells.

Finally, IFN-γ could have contributed to antiviral mechanisms *in vivo* by synergizing with other host-derived antiviral factors like TNF-α (Wong and Goeddel, 1986) and/or IFN-α (Fleischman *et al.*, 1984) and IFN-β (Zerial *et al.*, 1982). The antiviral activities of these cytokines are synergistic when acting together. However, experiments using antibodies to TNF-α or IFN-β have provided no evidence to suggest an essential role for either of these factors in mediating recovery of nude mice from infection with VV-HA-IL2.

Figure 1. ***Histologic changes in skins and livers of athymic, nude mice infected with recombinant VV and treated with anti-IFN-γMab.***

Haematoxylin and eosin-stained histologic sections of skins and livers taken 5 days after i.v. injection with 10^7 PFU of VV-HA-IL2 or VV-HA-TK and treated with anti-IFN-γ Mab or otherwise. Red blood cell extravasation [arrows] was often observed in the dermal interstitial spaces in skin sections of mice inoculated with VV-HA-IL2 and treated with Mab (a), but not in sections from mice given VV-HA-IL2 only (b). [magnification x 100] Massive mononuclear cell [MN] infiltration and necrotic lesions [N] were typically seen in livers of mice infected with VV-HA-IL2 and treated with the Mab (c). Livers from mice infected with VV-HA-TK and treated with the Mab had significantly smaller numbers of mononuclear cells [MN] in the foci (d). [magnification x 80]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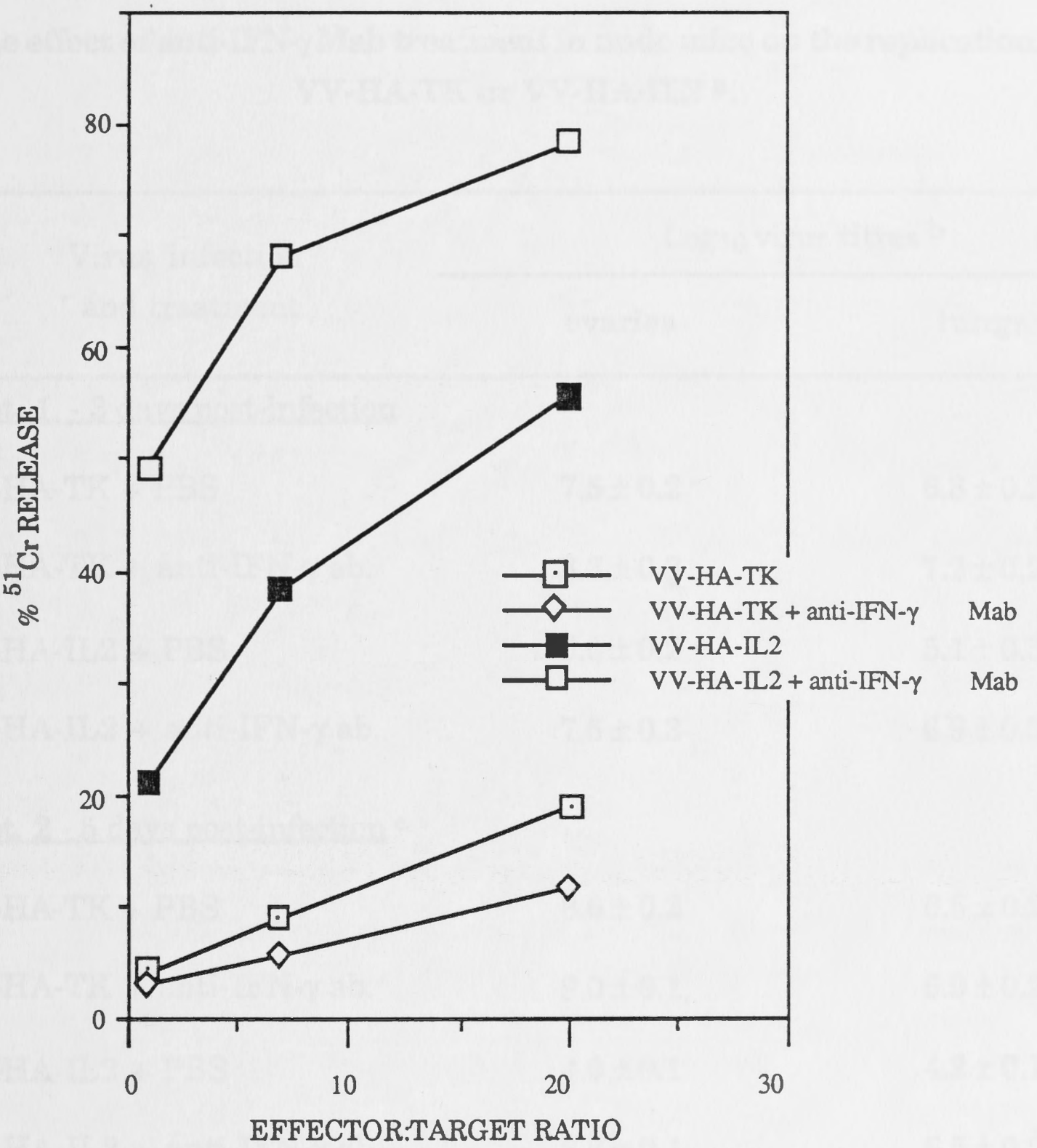


Figure 2. ***The effect of IFN-γ Mab treatment on splenic NK cell activity*** **.**

Six to 8-week old female nude mice were given 500U of anti-IFN-γ Mab i.p. on days -1, 0, 1, 2 and 3. Mice were infected i.p. with 10^7 PFU virus on day 0, and 5 days later the splenic cytolytic activity on YAC-1 targets was determined using pooled splenocytes. The assay was carried out in triplicate for each effector to target ratio and SEM were less than 5%.

Table 1.

The effect of anti-IFN-γ Mab treatment in nude mice on the replication of VV-HA-TK or VV-HA-IL2 [a].

Virus infection and treatment	Log_{10} virus titres [b]	
	ovaries	lungs
Expt. 1. - 3 days post-infection		
VV-HA-TK + PBS	7.5 ± 0.2	6.3 ± 0.2
VV-HA-TK + anti-IFN-γ ab.	8.3 ± 0.3	7.2 ± 0.2
VV-HA-IL2 + PBS	5.0 ± 0.2	5.1 ± 0.3
VV-HA-IL2 + anti-IFN-γ ab.	7.5 ± 0.3	6.9 ± 0.3
Expt. 2 - 5 days post-infection [c]		
VV-HA-TK + PBS	8.6 ± 0.2	6.5 ± 0.2
VV-HA-TK + anti-IFN-γ ab.	9.0 ± 0.1	6.9 ± 0.2
VV-HA-IL2 + PBS	4.0 ± 0.1	4.2 ± 0.1
VV-HA-IL2 + anti-IFN-γ ab.	8.8 ± 0.1	6.5 ± 0.2

[a] Six week old female nude mice were given 500U of anti-IFN-γ Mab i.p. on days -1, 0, 1 and 2. Mice were infected i.v. with 10^7 PFU virus on day 0, and 3 days later mice were sacrificed and virus titres in organs were determined.

[b] Data shown are the geometric means of log_{10} virus titres ± SEM of 4 individual lungs or pairs of ovaries.

[c] The regime of Mab treatment and infection with virus for Expt. 2 is as for [a] above except that mice were given the Mab on day 3 as well.

Table 2.

Mortality in nude mice treated with anti-IFN-γ antibody and infected with VV-HA-IL2. [a]

Virus and/or antibody	No. of mice	% Mortality	MTD [b] (death on days p.i.)	
antibody only	3	nil	-	
VV-HA-TK	5	100	15.4	(12,14,15,18,18)
VV-HA-TK + antibody	5	100	14.6	(13,13,14,16,17)
VV-HA-IL2	5	nil	-	
VV-HA-IL2 + antibody	5	100	9.6	(5,6,7,13,17)

[a] Ten week old female nude mice were given 500U of antibody i.p. on days -1, 0, 1, 2 and 3. Mice were infected i.v. with 10^7 PFU of virus on day 0. Control mice were left uninfected. One U of antibody neutralizes 10U of the anti-viral activity of IFN-γ *in vitro*.

[b] Mean time to death.

Table 3.

The effect of increasing anti-IFN-γ antibody concentration on mortality of nude mice infected with VV-HA-IL2.[a]

No of mice	Ab. conc.[b]	Infected/ Uninfected	MTD [c] (death on days p.i.)	
4	100U	VV-HA-IL2	11.00	(9,11,11,13)
2	100U	-	-	
4	200U	VV-HA-IL2	9.75	(7,9,10,13)
2	200U	-	-	
4	300U	VV-HA-IL2	8.50	(7,8,9,10)
2	300U	-	-	
4	400U	VV-HA-IL2	7.75	(6,7,8,10)
2	400U	-	-	
4	500U	VV-HA-IL2	6.00	(5,5,6,8)
2	500U	-	-	

[a] Six week old female nude mice were given i.p. varying concentrations of anti-IFN-γ on days -1, 0, 1, 2 and 3. Mice were infected on day 0 with 10^7 PFU virus i.v. and controls were left uninfected.

[b] One U of antibody neutralizes 10U of the antiviral activity of IFN-γ in vitro.

[c] Mean time to death.

Table 4.

Phenotype of peritoneal cytolytic effector cells from VV-HA-IL2-infected and anti-IFN-γ Mab-treated nude mice. [a]

Treatment	E:T	% specific lysis of YAC-1 targets: effectors from VV-HA-IL2-infected, antibody-treated mice	% specific lysis of YAC-1 targets: effectors from VV-HA-IL2-infected mice
C	10:1	78	21
	3:1	66	13
	1:1	49	6
C + anti-Thy1.2	10:1	67	15
	3:1	43	8
	1:1	32	3
C + anti-as-GM_1	10:1	69	17
(1:50 dilution)	3:1	51	7
	1:1	38	5
C + anti-as-GM_1	10:1	36	4
(1:5 dilution)	3:1	24	1
	1:1	15	0

[a] Six week old female nude mice were given 500U of anti-IFN-γ Mab i.p. on days -1, 0, 1, 2 and 3, and infected i.v. with 10^7 PFU of VV-HA-IL2 on day 0. Peritoneal cells were harvested 5 days after infection from groups of 4 mice, treated or untreated with anti-IFN-γ Mab. Aliquots of $1x10^6$ peritoneal cells were treated with appropriate antibody and/or complement (C) and effectors were used in a cytotoxicity assay. Anti-as-GM_1 was used at 2 dilutions, and by increasing the concentration more of the cytotoxic activity on YAC-1 targets could be abrogated.

Table 5.

The effect of exogenous rIL-2 or rIFN-γ administration on survival of nude mice infected with VV-HA-TK.

No. of mice	Virus [a]	Treatment [b]	% Survival	MTD [c] (Death on days p.i.)
3	nil	rIL-2	100	-
3	nil	rIFN-γ	100	-
5	VV-HA-IL2	-	100	-
5	VV-HA-TK	-	0	12.2 (9,11,12,14,15)
5	VV-HA-TK	rIL-2	0	23.4 (19,21,24,25,28) [d]
5	VV-HA-TK	rIFN-γ	0	25.8 (18,23,27,28,33) [d]

[a] Eight week old male nude mice were given 10^7 PFU virus i.v.

[b] One hour after infection, mice were given i.p. 600U of rIL-2 or rIFN-γ (+) and thereafter every 8 hours for a period of 5 days. Controls were given PBS only (-).

[c] Mean time to death.

[d] Significant, $p < 0.001$ - Students T-test, compared to group given VV-HA-TK only.

Table 6.

The effect of macrophage depletion on mortality and VV-HA-IL2 clearance in nude mice.

No of mice	Virus [a]	Macrophage [b] depletion	% Survival	MTD [c] (Death on days p.i.)
(i) Mortality				
5	VV-HA-TK	-	0	13.4 (10,13,14,15,15)
5	VV-HA-TK	+	0	14.0 (10,10,14,18,18)
5	VV-HA-IL2	-	100	-
5 [d]	VV-HA-IL2	+	60	30 (30,30)

(ii) Viral titres [d]	Log_{10} virus titres ± SEM	
	Lungs	Brain
	3.8 ± 0.4	4.4 ± 0.4

[a] Eight week old male nude mice were given 10^7 PFU virus i.v.

[b] Mice were depleted (+) of macrophages as described earlier. Control mice were left untreated (-) with silica .

[c] Mean time to death.

[d] The 3 surviving mice were sacrificed on day 32 p.i. and virus titres in lungs and brain were determined.

Table 7.

Anti-IFN-γ Mab treatment in normal CBA/H mice : Effect on NK cell responses and virus titres. [a]

Infection and Treatment	E:T	% Specific lysis of YAC-1 targets	Log_{10} virus titres ± SEM [b]	
			ovaries	lungs
VV-HA-TK	20:1	37	7.9 ± 0.1	4.7 ± 0.1
	7:1	19		
	2:1	7		
VV-HA-TK+ IFN-γ Mab	20:1	45	8.8 ± 0.2 [c]	5.7 ± 0.3 [c]
	7:1	25		
	2:1	13		
VV-HA-IL2	20:1	52	3.9 ± 0.1	3.8 ± 0.1
	7:1	33		
	2:1	17		
VV-HA-IL2 + IFN-γ Mab	20:1	61	7.6 ± 0.2 [d]	5.3 ± 0.1 [d]
	7:1	49		
	2:1	30		

[a] Six week old female CBA/H mice were treated with 300U anti-IFN-γ Mab i.p. on days -1, 0, 1 and 2. Mice were infected with 10^7 PFU virus on day 0, and 3 days later, splenocytes were used to measure NK activity while ovaries and lungs were used for determination of viral titres.

[b] Data shown are the geometric means of log_{10} virus titres ± SEM of pairs of ovaries and individual lungs from groups of 3 mice.

[c] Significant, $p < 0.01$, Students T-test.

[d] Significant, $p < 0.001$, Students T-test.

Table 8.

Anti-IFN-γ Mab treatment in normal CBA/H mice : Effect on Tc cell responses and virus titres [a].

Infection and Treatment	E:T	% Specific lysis of VV-WR-infected L929 targets [b]	Log_{10} virus titres ± SEM in ovaries [c]
VV-HA-TK	20:1	66	6.5 ± 0.2
	7:1	54	
	2:1	26	
VV-HA-TK + IFN-γ Mab	20:1	82	7.5 ± 0.1
	7:1	67	
	2:1	58	
VV-HA-IL2	20:1	60	< 2.0
	7:1	45	
	2:1	21	
VV-HA-IL2 + IFN-γ Mab	20:1	75	5.6 ± 0.1
	7:1	65	
	2:1	48	

[a] Six week old female CBA/H mice were treated with 300U anti-IFN-γ Mab i.p. on days -1, 0, 1, 2, 3 and 4. Mice were infected with 10^7 PFU virus on day 0, and 6 days after infection, splenocytes were used to measure Tc activity, while virus titres in ovaries and lungs were determined.

[b] The levels of lysis shown are lysis of virus-infected targets minus lysis of uninfected targets. Lysis of uninfected targets was between 5-10% of the levels of lysis seen on VV-WR-infected targets

[c] Data shown are the geometric means of log_{10} virus titres ± SEM of pairs of ovaries from 3 mice per group.

Table 9.

Depletion of CD4+ or CD8+ cells in normal mice : Effect on VV-HA-IL2 replication.

Virus infection and antibody treatment [a]	Geometric means of log_{10} virus titres ± SEM [b]	
	ovaries	lungs
VV-HA-IL2	4.1 ± 0.1	3.8 ± 0.2
VV-HA-IL2 + anti-CD4	5.5 ± 0.1 [c]	5.1 ± 0.2 [c]
VV-HA-IL2 + anti-CD8	4.2 ± 0.2	4.1 ± 0.2

[a] Eight week old female CBA/H mice were treated with anti-CD4 or anti-CD8 Mab to specifically deplete CD4+ and CD8+ T cells as described under Materials and Methods. Three days after infection, mice were sacrificed for the determination of virus titres in organs.

[b] The datd shown are the geometric means of log_{10} virus titres ± SEM of paired ovaries or indiviual lungs from 4 mice per group.

[c] Significant, $p < 0.001$ Students T-test, when compared with VV-HA-IL2 titres in untreated mice.

CHAPTER 6

VV growth in murine ovaries: A functional and histological analysis.

INTRODUCTION

Acceptance of a vaccine by any community is governed by a complex equation, balancing efficacy against safety, gain against risk and fear of disease against the potential side effects associated with vaccination. The incidence of encephalitis and other complications following vaccination with existing strains of VV (approximately 1:10,000) was judged to be unacceptable in the western countries in the final years before smallpox was eradicated (Mims and White, 1984d). Because the standards have changed for human vaccines, the smallpox vaccine, which has remained more or less unchanged for more than a hundred years, would never have been licensed if introduced a few years ago (Mims, 1987b). Since recombinant VV have been proposed as live virus vaccines for human and veterinary use, it is crucial that existing strains are improved with regard to safety.

While the primary aim of inserting the IL-2 gene into the VV genome was to enhance the immune responses to VV and the co-expressed influenza HA in immunized mice, there is sufficient evidence to conclude that the expression of this lymphokine during infection with VV-HA-IL2 reduced virulence. Data presented earlier clearly indicated that in mice treated with anti-IL-2 (Chapter 2, Table 3) or anti-IFN-γ Mab (Chapter 5, Tables 1, 3 and 5), titres of VV-HA-IL2 were increased significantly compared to growth in untreated controls, and sometimes comparable with titres of VV-HA-TK.

This final experimental chapter addresses essential findings related to the safety and potential use of VV as a live virus vector to deliver foreign antigens of medical and verterinary importance. The first of these is the highly productive infection caused by VV in ovaries of mice described in the earlier chapters. Using normal mice, the effect of VV replication on the reproductive function of ovaries was investigated. Since VV-HA-TK had been constructed from VV-WR, the capacity for VV-WR to replicate in ovaries was studied. It was also of interest to determine if human vaccine strains of VV replicated in murine ovaries. After all, VV-WR is a mouse-adapted virus derived from the human vaccine strain VV-NYBH. Three human vaccine strains with varying degrees of pathogenicity (reviewed in Fenner *et al.*, 1988c) were used in the study.

The second important finding relates to the fact that VV-HA-IL2 was cleared more rapidly than VV-HA-TK from ovaries and other organs of normal mice without affecting immunogenicity. The use of recombinant VV-encoding immunomodulatory molecules such as IL-2 may serve to make vaccines safer. The effect of IL-2-induced mechanisms of rapid viral clearance on the reproductive function of ovaries was investigated histologically and functionally.

Finally, during the course of this work, an unexpected finding was made. In order to show the causal relationship between VV-HA-IL2-induced NK cells and their antiviral function *in vivo*, normal mice were depleted of NK (as-GM_1^+) cells with anti-as-GM_1. Instead of exacerbating the infection, treatment with anti-as-GM_1 reduced significantly VV-HA-TK and VV-HA-IL2 titres in ovaries of infected mice. However, lung viral titres in antiserum treated mice increased significantly (data not shown). Evidence for the role of NK cells in contributing to antiviral function *in vivo* was nevertheless obtained using a specific Mab (anti-NK1.1) to NK cells. In an attempt to understand the mechanism behind lower viral titres in ovaries of anti-as-GM_1-treated mice, some preliminary work was carried out. The results are discussed in the context of viral tropism and how knowledge of the passage history of any strain being considered as a candidate vaccine may be crucial to its selection.

MATERIALS AND METHODS

Mice.

Specific pathogen-free mice were used at 6-8 weeks of age. The strains used in the present study, i.e., BALB/c, C57BL/6J, C3H/HeJ, CBA/H, DBA/2J and SJL/J were all obtained from the Animal Services Section, JCSMR.

Viruses.

The mouse brain passaged VV-WR and the recombinant viruses VV-HA-TK and VV-HA-IL2 have been described in Chapter 2. Three human vaccine strains of VV were also used. The New York City Board of Health strain (VV-NYBH), also known as the Wyeth strain, and the Lister (Elstree) strain (VV-Lister or VV-Elstree) were obtained from Dr. D. B. Boyle, Australian Animal Health Laboratory, Geelong, Victoria, Australia. The Copenhagen strain (VV-Copenhagen) was kindly provided by Dr. M.-P. Kieny, Transgene, Strasbourg, France. The 3 strains may be classified in terms of pathogenicity in mice, rabbits and irradiated rats as follows: mildly pathogenic - VV-NYBH; moderately pathogenic - VV-Lister; and highly pathogenic - VV-Copenhagen (reviewed in Fenner *et al.*, 1988c). This classification also reflected the frequency of complications after vaccination with any one of the 3 strains.

Treatment of normal mice with antiserum to as-GM_1.

Female CBA/H mice were given the anti-as-GM_1 antiserum i.v. in a 150µl volume (stock antiserum diluted 1:5) on 2 alternate days prior to immunization (day 0), and thereafter everyday except on the day when mice were sacrificed for determination of viral titres.

Establishment of primary and secondary murine ovarian cell culture

Paired ovaries from 20 CBA/H or C57BL/6J mice were aseptically removed and minced with sharp surgical scissors. Minced tissue was incubated in 2ml of HBSS containing collagenase (2mg/ml) (Boehringer-Mannheim, Tutzig, FRG) at 37°C. After 20 min, the digested ovarian tissues were gently pressed through stainless steel mesh using syringe plungers, clumps removed by passage through nylon mesh and the resulting cell suspension was washed once with HBSS and then with

RPMI 1640 (Gibco Laboratories, Grand Island, NY., USA) containing 5% FCS. After the final wash, $3x10^6$ viable cells were seeded into 175 cm^2 Nunclon tissue culture flasks in 50ml of RPMI 1640 containing 20% FCS and maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 24h, dead cells were removed by gentle aspiration and fresh RPMI 1640 containing 15% FCS (complete RPMI) was added to the primary cultures. Ovarian cells were used after a further passage (secondary culture). By this time, most of the cells had a fibroblast-like morphology. The cells were used for virus replication studies *in vitro* and flow cytometry.

Flow cytometry.

As described in Chapter 4.

RESULTS

Preferential growth of VV in ovaries.

In Chapter 2, both VV-HA-TK and VV-HA-IL2 were shown to replicate in murine ovaries. Since both these recombinants were constructed from VV-WR, it was of interest to investigate whether the wild type virus also caused a productive infection in ovaries. Normal CBA/H mice were therefore infected i.v. with 10^7 PFU of VV-WR in order to compare virus replication in ovaries versus growth in other visceral organs. Groups of 4 mice were sacrificed on days 1, 2 and 3 after infection and virus titres were determined in the ovaries, lungs, liver and spleen. Liver and spleen had the lowest titres, reaching $10^{5.3}$ PFU by day 3. The mean lung viral titre was $10^{6.4}$ PFU on day 3 but ovaries had the highest mean titres on all 3 days of assay, rising from $10^{5.0}$ PFU on day 1 to $10^{7.3}$ PFU on day 2 and reached $10^{8.6}$ PFU on day 3 (Table 1). Since ovaries weigh approximately 14 times less than lungs, virus titre per gram of tissue was about 2,000-fold higher in ovaries than in lungs. The preferential growth of VV-WR in ovaries suggested tissue-specific tropism and/or extremely productive infection of ovarian cells.

The ability of other VV strains to replicate in ovaries.

VV-WR is a mouse brain passaged, mouse-adapted, neurovirulent virus derived from VV-NYBH. The human vaccine strain VV-NYBH had been widely used during the smallpox eradication campaign. Therefore a study to test the ability of 3 human vaccine strains, VV-NYBH, VV-Elstree and VV-Copenhagen to replicate in ovaries was undertaken. Virus titres of paired ovaries from groups of 3 CBA/H mice inoculated i.v. 3 days previously with 10^7 PFU of either VV-WR, VV-NYBH, VV-Elstree or VV-Copenhagen were determined. The mean VV-WR titre was 4-5 $\log_{10}$ higher than the other three strains (Table 2). The ability of the 3 human vaccine strains to replicate in ovaries correlated with the pathogenicity of these strains for mice and rabbits (Table 2, and Fenner *et al.* 1988b). VV-WR also replicated efficiently in ovaries after i.p. inoculation but not after s.c. inoculation into the foot-pad (data not shown).

VV replication in ovaries of various mouse strains.

The ability of VV-HA-TK and VV-HA-IL2 to replicate in ovaries of various mouse strains was next tested. Groups of 4 female BALB/c, C3H/HeJ, C57BL/6J, CBA/H, DBA/2J and SJL/J mice were given i.v. 10^7 PFU virus and 3 days later virus titres in ovaries were determined. VV-HA-TK was recovered from ovaries of all mouse strains tested with mean titres ranging from $10^{7.0}$ to $10^{8.2}$ PFU (Table 3). On the other hand, mean VV-HA-IL2 titres ranged from $10^{3.9}$ to $10^{5.1}$ PFU.

Histologic changes associated with VV replication in ovaries.

The effect of VV replication in ovaries was investigated histologically. Haematoxylin and eosin stained sections of ovaries were obtained from CBA/H mice 1, 2, 3, 6 and 12 days after inoculation with 10^7 PFU of either VV-HA-TK, VV-HA-IL2 or VV-WR. Histological examination of infected ovaries indicated that VV-HA-IL2 had hardly replicated (Fig. 1a) and no damage was detectable, consistent with the low levels of virus recovered (Chapter 2, Fig. 4). In contrast, VV-HA-TK initially infected ovarian stroma, not follicles, and had destroyed many stromal cells by day 3 after infection. By day 6 post-inoculation with VV-HA-TK, the ovarian stroma had been largely destroyed (Fig. 1c) but by day 12, VV-HA-TK had completely destroyed the stroma and ovarian follicles (Fig. 1d). Histologic changes in ovaries after infection with VV-WR was comparable to that caused by VV-HA-TK (data not shown).

Ovarian damage and sterility in mice.

In order to determine whether the ovaries of mice infected with VV-WR or VV-HA-TK were functionally impaired, female CBA/H mice that had been inoculated 30 days previously with any one of the wild type or recombinant VV were individually mated with a male mouse and observed for 60 days or until they produced a litter.

All mice that had been infected either i.p. or i.v. with VV-WR did not produce litters over the 60 day observation period (Table 4). In the group that had been inoculated with VV-HA-TK, only 30% produced litters and these were delayed well beyond the normal gestation period of 20-21 days. Infection with VV-Copenhagen also reduced fertility; 70% of the mice produced litters and some of the mice that did become pregnant took

longer to do so (Table 4). In contrast, all mice that had been infected with VV-HA-IL2, VV-NYBH or VV-Elstree produced litters within the normal gestation period. These results indicate that obvious functional impairment of ovaries occurs when the VV strain has the capacity to replicate to high titres (e.g. 8 $\log_{10}$) in these organs. Because VV-HA-IL2 reaches maximal titres of only 4-5 $\log_{10}$ in infected ovaries (Chapter 2, Fig. 4), functional impairment is not detectable.

Treatment with anti-as-GM_1 reduces the capacity for VV to replicate in ovaries.

The antiserum to as-GM_1 was initially used in an attempt to deplete as-GM_1^+ NK cells in normal mice but was found to be unsuitable (see Chapter 4). Although the antiserum treatment resulted in enhanced viral titres in lungs of normal mice (data not shown), ovarian VV titres were markedly reduced. The results from a representative experiment are shown in Table 5. In that experiment, normal CBA/H mice treated with anti-as-GM_1 were infected i.v. with 10^7 PFU of VV-HA-IL2 and 3 days later, virus titres in ovaries were determined.

While the mean VV-HA-IL2 titre in ovaries of untreated CBA/H mice 3 days after infection was 4.5 $\log_{10}$, virus was below the level of detection (< 2 $\log_{10}$ PFU) in antiserum-treated mice. Treatment with anti-as-GM_1 also resulted in a 1.5 $\log_{10}$ reduction in VV-HA-TK titre in ovaries (Table 5). Using a specific Mab to NK cells (anti-NK-1.1), evidence was presented in Chapter 4 which indicated that NK cell depletion in normal mice resulted in enhanced ovarian and lung VV-HA-IL2 titres (see Chapter 4, Table 3). Further investigation of the phenomenon whereby anti-as-GM_1 reduced capacity for VV to replicate in ovaries using cultured ovarian fibroblasts *in vitro* was unsuccessful. Although VV-WR, VV-HA-TK and VV-HA-IL2 replicated successfully in cultured ovarian cells, treatment of the cells with antiserum prior to infection with VV was found to be toxic to the cells at the various concentrations used (data not shown). The toxicity may not, however, have reflected the *in vivo* phenomenon since histological examination of ovaries from anti-as GM_1 treated mice that had been infected with VV-HA-TK indicated that the ovarian stroma was not as badly damaged as was apparent in mice that had been inoculated with VV-HA-TK but not treated with antiserum. *In vitro*, incubation of

VV-HA-TK or VV-HA-IL2 with high concentrations of anti-as-GM_1 for 1 h prior to titration had no effect on viral titres (data not shown).

Murine ovarian cells express as-GM_1 or a closely related epitope.

The above findings suggested that anti-as-GM_1 prevented VV infection of the ovarian cells. One possible explanation was that the ovarian cells expressed a cell surface epitope identical to or cross-reactive with as-GM_1, and following treatment with anti-as-GM_1 *in vivo*, binding of the antibody to the ovarian cell surface may have impeded virus binding or entry to target cells. The presence or absence of "as-GM_1" on ovarian cells was tested by flow cytometry. Freshly prepared ovarian cells had low viability and were therefore unsuitable for cytofluorometry. Cultured CBA/H ovarian cells were stained with anti-as-GM_1 as the primary antibody followed by FITC-conjugated secondary antibody. Flow cytometric analysis revealed that more than 90% of CBA/H ovarian cells expressed varying levels of 'as-GM_1' (Fig. 2). Similar findings were also made with cultured C57BL/6J ovarian cells (data not shown). The possibility that levels of as-GM_1 on the ovarian cells could have increased after culture cannot be excluded.

DISCUSSION.

VV replication in murine ovaries has not been documented previously. Data presented in this chapter and Chapter 2 show that in normal mice VV-WR and VV-HA-TK, a recombinant virus constructed from VV-WR, replicated to substantially higher titres in ovaries than in lungs, liver and spleen. VV-WR also replicated more efficiently in ovaries than the vaccine strains VV-NYBH, VV-Elstree and VV-Copenhagen. The recombinant viruses, VV-HA-TK and VV-HA-IL2 also seeded and successfully replicated in ovaries of 6 different mouse strains tested although VV-HA IL2 titres were consistently lower.

Histological examination indicated that infection with VV-WR or VV-HA-TK led to the destruction of ovarian stroma and eventually follicles. Consistent with histological evidence, infection with VV-WR i.p. or i.v. resulted in 100% sterility, thereby indicating the functional impairment of ovaries. VV-HA-TK infection resulted in a 70% sterility in mice and this may be a reflection of the reduced virulence of VV-HA-TK compared to VV-WR discussed in Chapter 2.

Of the 3 vaccine strains tested, only VV-Copenhagen caused any infertility (30%) in infected mice. VV-Copenhagen grew to higher titres in ovaries than VV-NYBH or VV-Elstree and has been reported (Fenner *et al.*, 1988c) to be associated with post-vaccination complications in human vaccinees. The inability of the human vaccine strains to replicate as efficiently as VV-WR in ovaries and the finding that s.c. inoculation did not result in infection of ovaries may perhaps explain why no adverse effects on fertility of vaccinees immunized against smallpox were reported during the smallpox eradication program. However, viraemia has been noted after primary vaccination and virus was recovered from tonsillar and pharyngeal swabs (Fenner *et al.*, 1988d), thus providing the opportunity for virus to spread to ovaries. It is possible that the ovarian tissue-specific tropism seen in mice does not exist in humans. Interestingly, VV-WR was found to replicate in ovaries of rats (D.Willenborg, personal communication) and rabbits (unpublished observations).

As stated earlier, VV-WR was derived from VV-NYBH by mouse brain passage and selected for its neurovirulence (neurovaccinia). The adaptation to mouse could explain, at least in part, the efficient

replication of VV-WR but not VV-NYBH in mouse ovaries. In this regard, it is interesting to note that the glycosphingolipid, as-GM_1 (or a cross-reacting epitope), is expressed on at least 2 strains of mouse ovarian cells and that the antiserum, anti-as-GM_1, reduced the capacity for VV to replicate in ovaries. The anti-as-GM_1 used in the present study had been prepared by immunisation of rabbits with purified as-GM_1 (Kasai *et al.*, 1980), which itself had been prepared from the naturally occuring ganglioside, GM_1, in bovine brain. Antiserum to as-GM_1 prepared in this way has been reported to exhibit a small degree of cross-reactivity with GM_1 (Kasai *et al.*, 1980; Jacquemart *et al.*, 1988). GM_1 and related glycosphingolipids occur in high concentration in the central nervous system (CNS) (Svennerholm, 1972). Since repeated mouse brain passage of VV-NYBH, which is a dermotropic strain, allowed the derivation of VV-WR, a neurovaccinia virus, and since anti-as-GM_1 impeded VV infection of ovaries, it may be possible that tropism for both the CNS and ovaries was acquired by adaptation of the virus to bind to as-GM_1 or a closely related ganglioside or cell surface glycoproteins that have the same carbohydrate chain as those found on gangliosides (Tonegawa and Hakamori, 1977).

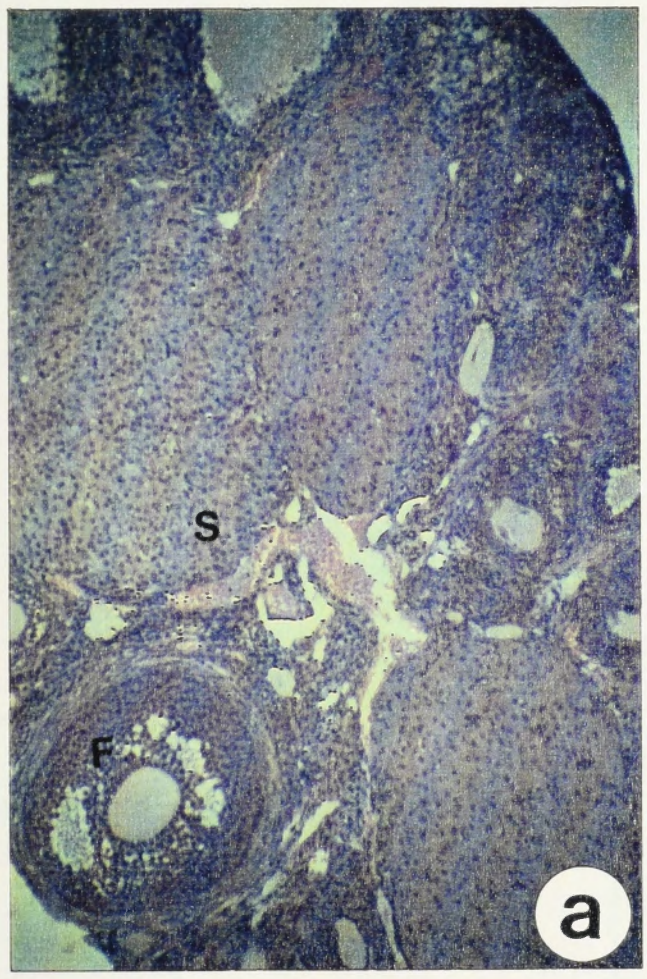
It is likely that viral tropism for any particular tissue is influenced by multiple surface markers expressed by cells that make up the tissue. Obviously, the receptor will be a major determinant of the characteristic viral tropism. However, additional factors are also believed to be involved in viral tropism and the expression of the relevant receptor may not make a target cell permissive to infection (Mims, 1989). The receptor for VV is thought to be the epidermal growth factor receptor (EGF-R) (Eppstein *et al.*, 1985) but reports by others (Stroobant *et al.*, 1985; Buller *et al.*, 1988) suggest that this may not be the only receptor. VV can successfully infect and replicate to comparable levels in EGF-R^+ and EGF-R^- cells (Stroobant *et al.*, 1985) suggesting a lack of any absolute requirement for EGF-R for the growth of VV in tissue culture. Possible mechanisms by which anti-as-GM_1 reduces the capacity for VV to infect murine ovaries could be by impeding viral binding to the cell surface or via co-capping of the EGF-R with as-GM_1 (or a cross-reactive epitope), thereby removing the viral receptor. Although further investigations are necessary, there is a distinct possibility that as-GM_1 or the cross-reacting epitope serves as an additional receptor for VV. In this regard, it relevant to note that rabies virus, which is also a neurotropic virus, may bind to acetylcholine

receptors at the neuromuscular junction (site of initial infection), but at a later stage to ganglioside receptors on neural cells (Conti *et al.*, 1988).

Data presented in this chapter underscore 3 important points with regard to the use of VV as a live virus vaccine vector in humans or animals. Firstly, VV has been shown to replicate in mouse ovaries and cause infertility. Secondly, inclusion of the IL-2 gene markedly reduced the extent of viral replication and ovarian damage and prevented infertility. The third point relates to the choice of appropriate VV strains for use as recombinant vaccines. The passage of history of the strain being considered will have to be thoroughly investigated to avoid any undesirable side effects caused by tissue tropism. It must be stressed however, that sterility in mice was only observed when virus was inoculated i.v. or i.p. but not s.c. Vaccination against smallpox using VV had been carried out only s.c. by scarification, presumably reducing the risk of spread to ovaries, even if the tropism existed.

Figure 1. ***Histologic changes in ovaries of normal CBA/H mice infected with recombinant VV.***

Haematoxylin and eosin-stained histological sections of ovaries from normal CBA/H mice taken at various times after i.p. injection with 10^7 PFU of VV-HA-IL2 or VV-HA-TK. Histologic changes in ovaries of VV-HA-IL2-infected mice were hardly noticeable at day 3 post-infection and look normal (a). The rapid replication of VV-HA-TK had damaged the ovarian stroma [S] partially at day 3 post-infection (b). Six days after infection, VV-HA-TK had destroyed large areas of the stroma (c) but by day 12 post-inoculation, the ovarian stroma [S] and follicles [F] were completely destroyed (d). [magnification x100].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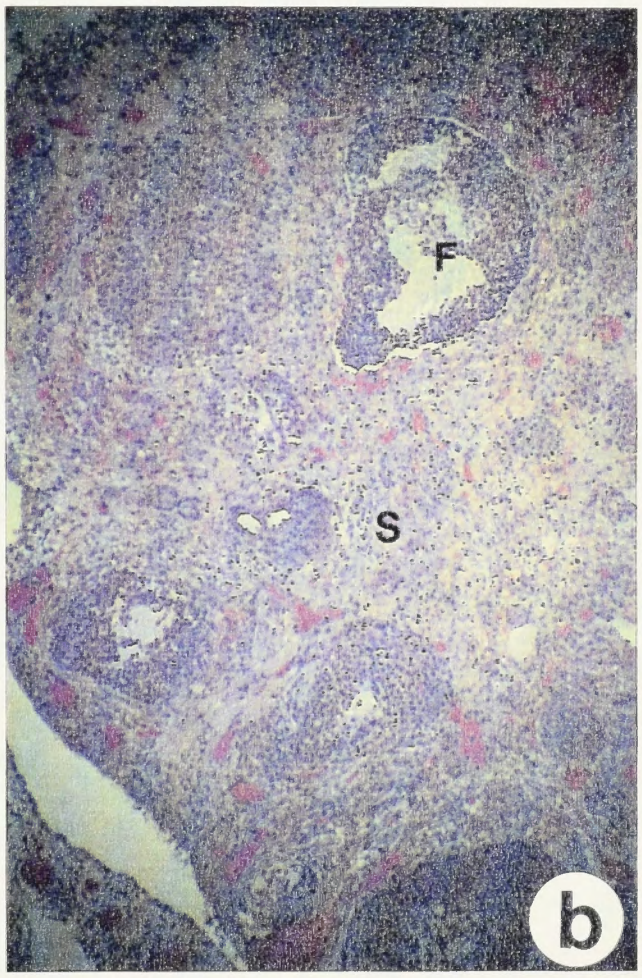
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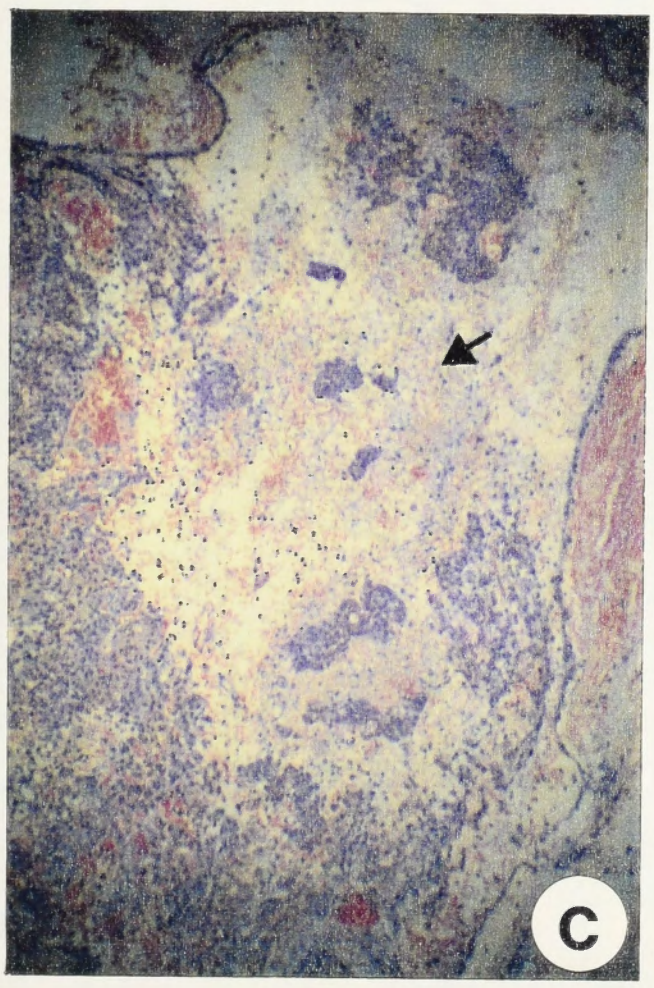
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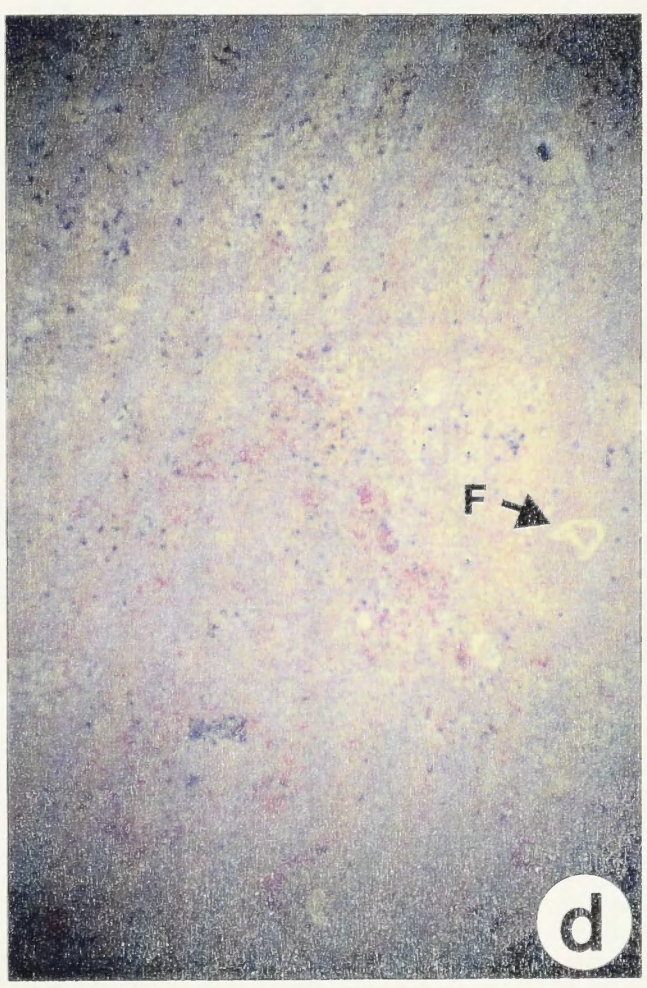
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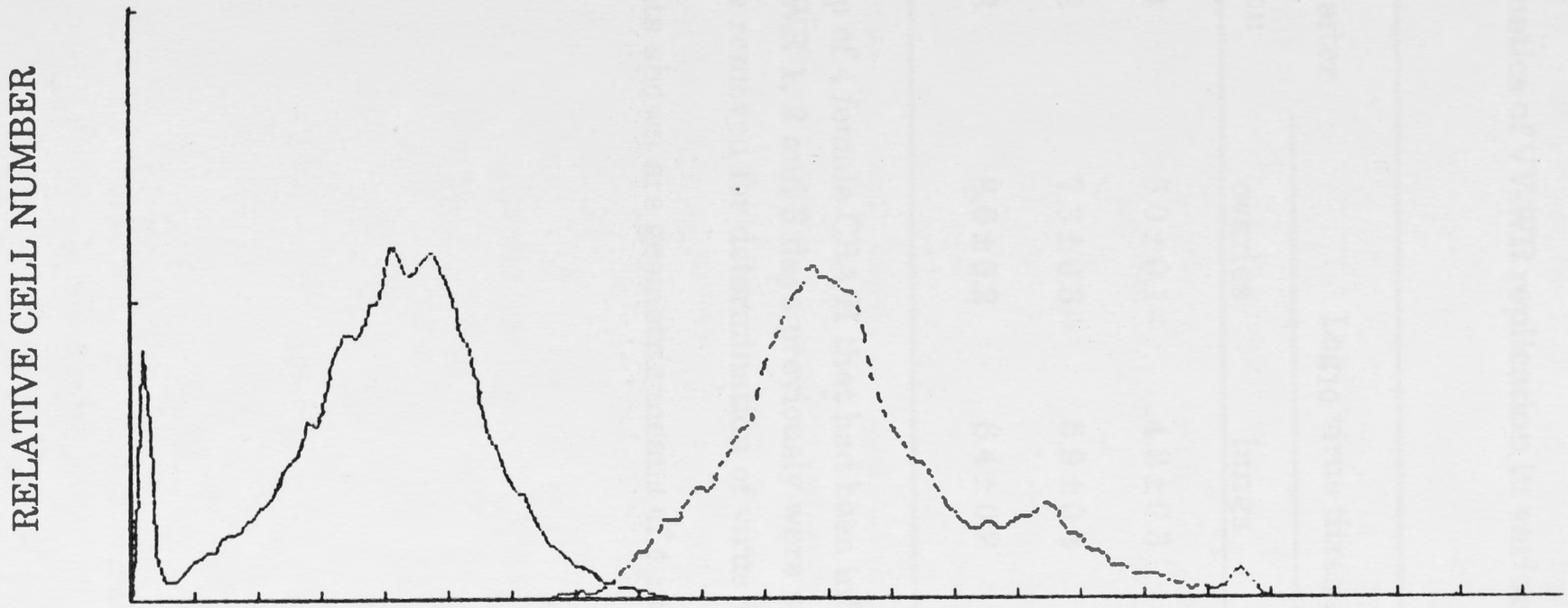

Figure 2. ***Expression of as-GM$_1$ on CBA/H ovarian fibroblast cells detected by flow cytometry.***

One million cells were incubated either with the secondary FITC-conjugated antibody alone (continuous line) or with anti-as-GM$_1$ as primary antibody followed by secondary FITC-conjugated antibody (broken line). A total of 20, 000 fluorescent activated cell sorting events were analysed.

Table 1.

Kinetics of VV-WR replication in various organs of CBA/H mice. [a]

Day(s) after infection	Log_{10} virus titres in organs ± SEM [b]			
	ovaries	lungs	spleen	liver
1	5.0 ± 0.1	4.2 ± 0.3	3.8 ± 0.3	4.0 ± 0.4
2	7.3 ± 0.3	5.9 ± 0.4	5.0 ± 0.3	4.8 ± 0.2
3	8.6 ± 0.2	6.4 ± 0.2	5.3 ± 0.3	5.3 ± 0.2

[a] Group of 4 female CBA/H that had been infected i.v. with 10^7 PFU of VV-WR 1, 2 and 3 days previously were sacrificed and their organs were removed for determination of virus titres.

[b] Data shown are geometric means of 4 individual organ titres ± SEM.

Table 2.

Effect of VV strain on the ability to replicate in murine ovaries.

Virus strain	Description [a]	Log_{10} virus titres ± SEM [b]
i) VV-WR	mouse brain passaged neurovirulent	8.5 ± 0.2
ii) VV-Copenhagen	human vaccine highly pathogenic	4.5 ± 0.7
iii) VV-Elstree	human vaccine moderately pathogenic	3.9 ± 0.4
iv) VV-NYBH	human vaccine mildly pathogenic	2.8 ± 0.2

[a] The passage history and description of VV-WR is based on Wokatsch (1972). The classification of the 3 human vaccine strains with regards to pathogenicity in mice and which also reflects the severity of complications associated with vaccination using any of these strains is based on Fenner *et al.* (1988c).

[b] Data shown are geometric means of titres of paired ovaries from 3 individual animals ± SEM.

Table 3.

VV replication in ovaries of various mouse strains.

Mouse strain [a]	Log_{10} virus titres ± SEM [b]	
	VV-HA-IL2	VV-HA-TK
BALB/C	4.9 ± 0.3	7.8 ± 0.1
C3H/HeJ	4.5 ± 0.5	8.1 ± 0.1
C57BL/6J	3.9 ± 0.2	7.0 ± 0.1
CBA/H	4.7 ± 0.3	8.0 ± 0.1
DBA/2J	4.3 ± 0.3	8.2 ± 0.2
SJL/J	5.1 ± 0.3	7.7 ± 0.2

[a] Six to 8 week old female mice of the indicated strains were infected i.v. with 10^7 PFU virus and 3 days post-inoculation, pairs of ovaries were removed from groups of 4 mice for determination of virus titres.

[b] Data shown are the geometric means of titres of paired ovaries from groups of 4 mice ± SEM.

Table 4.

Effect of VV replication in ovaries on fertility of CBA/H mice.

Virus [a]	No. Pregnant/Total (%) [b]	Time to Delivery [c]
Nil	10/10 (100%)	20 - 21 [d]
VV-WR	0/20 (0%)	-
VV-Copenhagen	14/20 (70%)	20 - 28 [e]
VV-Elstree	10/10 (100%)	20 - 21 [d]
VV-NYBH	10/10 (100%)	20 - 21 [d]
VV-HA-TK	6/20 (30%)	26 - 33 [e]
VV-HA-IL2	20/20 (100%)	20 - 21 [d]

[a] Six week old CBA/H mice were inoculated i.v. with 10^7 PFU virus, except for VV-WR which was inoculated i.v. into 10 mice and i.p. into the remaining 10 mice.

[b] Thirty days after inoculation, mice were individually mated with a CBA/H male mouse and observed for a further 60 days or until they produced a litter.

[c] Time in days from exposure to male mouse to delivery of litter.

[d] Normal gestation period.

[e] Delay in gestation period.

Table 5.

The effect of treatment with anti-as-GM_1 antiserum on VV replication in ovaries of CBA/H mice.

Treatment with anti-as-GM_1 [a]	Log_{10} virus titres ± SEM [b]	
	VV-HA-IL2	VV-HA-TK
-	4.5 ± 0.3	7.3 ± 0.2
+	< 2.0	5.8 ± 0.2

[a] Paired ovaries were obtained 3 days after infection with VV-HA-IL2 or VV-HA-TK from groups of 4 mice that had been treated (+) or untreated (-) with anti-as-GM_1.

[b] Data shown are geometric means of titres of paired ovaries from groups of 4 mice ± SEM.

CHAPTER 7

DISCUSSION AND CONCLUDING REMARKS

Athymic nude mice lack mature T cells and are therefore unable to mount T-dependent immune responses. T cells are the principal producers of IL-2 which is pivotal for the generation and regulation of an immune response. Apart from its direct stimulatory effects on lymphoid and myeloid cells, IL-2 also regulates the production of other lymphokines like IFN-γ. The nude mouse model therefore provided an opportunity to study the function of VV-encoded IL-2 *in vivo* by analysis of the immune mechanisms induced by IL-2 that allowed their recovery from VV infection.

The highly 'attenuated' phenotype of VV-HA-IL2 in mice was due to antiviral mechanisms induced by virus-encoded IL-2. Data from experiments *in vivo* using Mabs to IL-2 or IFN-γ clearly established that VV-HA-IL2 could replicate to high titres in mouse tissues and therefore the reduced virulence did not result from the insertion of a foreign gene into the VV genome. Furthermore, the consequences of IL-2 production and subsequent activation of antiviral mechanisms following an infection with VV-HA-IL2 cannot be mimicked by the administration of exogenous IL-2 to mice infected with VV-HA-TK. It is the localized production of IL-2, at sites of viral infection, which makes VV-HA-IL2 a unique virus-infection model.

Evidence presented earlier suggested that the mechanisms operative between days 1-4 after infection in both nude and normal mice and which led to the rapid elimination of VV-HA-IL2, are similar. Data from studies using nude, normal and beige mice indicated clearly that multiple antiviral mechanisms were involved. A number of different classes of cytolytic cells, mononuclear phagocytes and IFN-γ were implicated as potential participants in the process of rapid viral elimination and recovery of nude mice but the relative importance of any one of these components was not easy to define.

Several lines of evidence suggested that NK cells contributed to clearance of virus early in infection. First, the kinetics of enhanced NK cell cytolytic activity in spleen, liver and ovary of nude and/or normal mice coincided with the rapid clearance of VV-HA-IL2 from these organs. Second, the clearance of VV-HA-IL2 in NK-cell deficient beige mice was significantly delayed compared to normal mice. Third, infiltration of mononuclear cells into foci of VV-HA-IL2 infection in the liver occurred more rapidly

than in VV-HA-TK-infected livers, with increased numbers of as-GM_1^+ cells in the former. Finally, VV-HA-IL2-induced cytolytic NK cells lysed VV-HA-IL2-infected targets more efficiently than VV-HA-TK-infected targets. These observations suggested that virus-encoded IL-2 activates and attracts NK cells to sites of viral replication where they exert their antiviral activity. That NK cells contributed to viral clearance was clearly demonstrated in experiments in which host NK cells were depleted with specific antisera or Mab.

In nude and normal mice, depletion of as-GM_1^+ or NK1.1$^+$ cells, respectively, resulted in enhanced VV-HA-IL2 titres, although no deaths were recorded in the former. As discussed in Chapter 4, it is possible that as-GM_1^+ cells were regenerated from as-GM_1^- progenitors in the presence of IL-2 or that other populations of IL-2 responsive antiviral effectors were expanded as a consequence of NK cell depletion. In normal mice, depletion of NK1.1$^+$ cells resulted in the emergence of an unidentified population of cytolytic cells which lysed both uninfected syngeneic and allogeneic targets *in vitro*. Whether this effector population, which apparently was expanded in the absence of NK cells, contributed to antiviral mechanisms is unclear. If this and other lymphoid or myeloid populations that were expanded and/or activated by virus-encoded IL-2 contributed to antiviral mechanisms, the relative contribution of NK cells in viral clearance becomes difficult to estimate.

In nude mice, a small proportion of splenocytes that lysed the classical NK targets, YAC-1, were as-GM1$^-$. There is a possibility that this population (presumably precursors of NK cells and/or T cells) contributed to the antiviral function.

Of the cytolytic cell populations obtained from ovaries of VV-HA-IL2-infected mice, one bore the phenotype of conventional NK cells and a second was as-GM_1^- and Thy1.2$^-$. The identity of the latter is not known but there is a distinct possibility that this cell type contributed to the process of virus elimination.

Data presented in Chapter 5 clearly established that IFN-γ played a crucial role in the clearance of VV-HA-IL2 both in nude and normal mice. Treatment of nude mice with a Mab to IFN-γ resulted in enhanced VV-HA-IL2 growth and inhibited recovery from infection. In normal mice, the Mab treatment also prevented the clearance of VV-HA-IL2.

However, the finding that this Mab also prevented clearance of VV-HA-TK further suggested that IFN-γ may play an important role in the normal antiviral mechanisms operative during VV infection. These findings suggested that virus-encoded IL-2 induced the secretion of IFN-γ by lymphoid cells in both nude and normal mice. *In vitro* analysis of nude mouse splenocytes indicated that both as-GM_1^+ and as-GM_1^- cells produced IFN-γ after exposure to IL-2. Since IFN-γ is a major contributor to antiviral mechanisms, its production by as-GM_1^- cells in nude mice depleted of as-GM_1^+ cells could explain why they survived infection with VV-HA-IL2. The as-GM_1^- cells could have represented precursors of NK and/or T cells.

In normal mice, the classes of lymphoid cells likely to have been triggered to secrete IFN-γ by virus-encoded IL-2 are T cells and NK cells. NK cells are known to produce IFN-γ after stimulation with IL-2 whereas T cells require antigen in addition to IL-2. In this regard, the *in vivo* depletion of NK-1.1$^+$ and CD4$^+$ T cells, but not CD8$^+$ T cells, resulted in enhanced levels of VV-HA-IL2 replication, as assessed three days after infection. The precise contribution of CD4$^+$ T cells to the clearance of VV-HA-IL2 is not known but there is a possibility that they were induced to produce IFN-γ by virus-encoded IL-2 and antigen.

IFN-γ could mediate antiviral activity in a number of ways which are not mutually exclusive. Firstly, IFN-γ produced early during the course of infection with VV-HA-IL2 may have induced an antiviral state in uninfected cells and thereby limited viral spread. Secondly, IFN-γ could have contributed to antiviral mechanisms by upregulating class I MHC and β2-m resulting in more efficient action of effector cells which recognize these molecules. As the class I MHC molecules complexed with viral peptides are recognized by CD8$^+$ Tc cells, this mechanism would only be applicable to normal mice. At 3 days post-infection, anti-VV Tc cell activity in VV-HA-IL2-infected mice was not detectable and depletion of CD8$^+$ T cells at this time did not affect viral titres in normal mice. Therefore, the concentration of class I antigens on target cells up to 3 days after infection with VV-HA-IL2 may not have influenced the efficiency of Tc cell recognition and elimination of infected cells. On the other hand, levels of expression of class I MHC-unassociated β2-m (a possible ligand for NK cells) could have influenced NK cell recognition and effector function (Mullbacher and King, 1989) in both nude and

normal mice. Treatment with a Mab to IFN-γ could potentially inhibit up-regulation of expression of the ligand and subsequently prevent binding and triggering of lysis of virus-infected cells. Indeed, treatment with the Mab in both nude and normal mice prevented the elimination of virus, although NK cell responses were higher than in control mice, thereby indicating that the effector function of NK cells was inhibited. Thirdly, IFN-γ could have mediated antiviral function through the activation of monocytes/macrophages. While the complete depletion of mononuclear phagocytes is not likely to be achieved *in vivo*, the data provided sufficient evidence which indicated that monocytes/macrophages (most likely activated by IFN-γ) contributed to the IL-2-induced antiviral mechanisms which enabled nude mice to recover from infection with VV-HA-IL2. The finding that silica treatment reduced NK activity allows the possibility that persistence of VV-HA-IL2 in silica-treated mice could have been partly due to impaired NK cell function *in vivo*. IFN-γ can only limit viral spread by virtue of its ability to protect uninfected cells from infection (apart from up-regulation of β2-m and class I MHC expression). On the other hand, macrophages (activated by IFN-γ) can phagocytose infectious virus particles released from infected cells or following NK cell-mediated lysis. Taken together, these findings imply that monocytes/macrophages are essential for the full expression of IFN-γ -mediated antiviral activity. However, because of the pleiotropic activity of this cytokine, the precise contribution to antiviral function *in vivo* cannot be established but it should be emphasized that IFN-γ acts in concert with other components of the immune system.

The mechanism(s) of recovery of athymic nude mice from VV-HA-IL2 infection can thus be summarised as follows. Virus-infected cells produce IL-2 which rapidly activates as-GM_1^+ (NK cells) and as-GM_1^- cells (precursors of NK and/or T cells; possibly precursors of spleen- and liver-associated macrophages). Indeed, elevated NK-like activity is detectable as early as 1 day post-infection. The chemotactic effect of IL-2 on NK cells (and possibly non-NK cells) allows recruitment of these effectors into sites of viral replication and this recruitment would precede activation if it were mediated by IL-2 from the foci of infection. Both as-GM_1^+ and as-GM_1^- cells are triggered by virus-encoded IL-2 to produce IFN-γ . IFN-γ activates mononuclear phagocytes (and presumably recruits blood monocytes). Activated NK cells, non-NK cells and the mononuclear

phagocytes, together with IFN-γ, contribute to the rapid elimination of VV-HA-IL2 and recovery of nude mice.

The need for antiviral $CD8^+$ Tc for recovery from a primary poxvirus infection is well established (Hirsch *et al.*, 1968; Blanden, 1970; 1971a, b; Buller *et al.*, 1987). The demonstration (Ruby and Ramshaw , submitted) that $CD8^+$ Tc cell fuction *in vivo* is dependent on IFN-γ further illustrates the importance of IFN-γ in antiviral immunity. The mechanisms of recovery described above involve 'non-specific' components of the immune system which can clearly be activated to mediate efficient antiviral functions to compensate for the absence of T-cell mediated mechanisms in nude mice.

Recovery from a generalized primary viral infection, e.g. mousepox, in normal mice and from VV-HA-IL2 infection in nude mice appear to involve similar but obviously not identical mechanisms. The roles of antiviral Tc cells, mononuclear phagocytes and IFN in the process of recovery from primary ectromelia virus infection were discussed by Blanden (1971b) and have been addressed in Chapters 1 and 5. I have presented evidence that recovery of athymic nude mice from VV-HA-IL2 infection is dependent on cytolytic NK-like or non-NK cells, mononuclear phagocytes and IFN-γ . Thus, a general model, based on that proposed by Blanden (1971b), encompassing three closely interacting components of the immune system may accomodate the data presented in this thesis. The model requires a cytolytic effector component (Tc cells in normal mice and NK and non-NK cells in nude mice) which produces IFN-γ (second component) and recruits mononuclear phagocytes (third component) to sites of viral replication. The cytolytic effectors lyse infected cells to prevent further viral replication (Tc cells can lyse infected targets before progeny virus is assembled, but this is not formally established for NK cells). IFN-γ -activated monocytes/macrophages phagocytose infectious virus particles and infected cell debris resulting from action of the cytolytic cells. IFN-γ induces an antiviral state in uninfected cells to prevent further viral spread and increases the expression of ligands (class I MHC for Tc cells and β2-m for NK cells) on target cells. It ought to be emphasised that in normal mice, antiviral Tc cells secrete IFN-γ after binding, via the TCR, to their ligands on virus-infected cells, whereas in nude mice, virus-encoded IL-2 triggers production of IFN-γ by NK and non-NK cells. Furthermore, for the former, secretion of IFN-γ by

antiviral Tc cells takes place only after Tc cell activation (a time-dependent process) whereas for the latter, IFN-γ secretion triggered by virus-encoded IL-2 (which itself is produced within a few hours after infection) occurs rapidly. The rapid activation of NK cells and secretion of IFN-γ influence the rapid elimination of VV-HA-IL2.

Inclusion of the IL-2 gene into the VV genome may provide a potentially safe vaccine vector which can be engineered to deliver foreign antigens of medical and verterinary importance. The virus is highly 'attenuated' and was shown to prevent undesirable side-effects in normal mice. Rapid elimination of virus did not result in reduced anti-VV Tc cell responses, although antibody titres to VV and influenza HA were lower in mice immunized with VV-HA-IL2 than in mice given VV-HA-TK (Andrew *et al.*, 1989). Nevertheless, immunization of normal mice with VV-HA-IL2 conferred levels of protection against challenge with the wild type, HA-homologous influenza virus comparable with the level of protection afforded by priming with VV-HA-TK (unpublished observations). Further investigations in this area should provide a better understanding of the effects of virus-encoded IL-2 on B- and T-cell memory which are relevant to long-term protection after vaccination.

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