GENERATION OF CELL-MEDIATED IMMUNE RESPONSES TO PICHINDE VIRUS IN MICE

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ABSTRACT

These studies were undertaken to characterize cytotoxic cell responses to Pichinde virus (PV; a member of the arenavirus family) in various strains of inbred mice. Emphasis was placed on examining the relationship between cells with natural killer (NK) activity and H-2 restricted, virus-specific cytotoxic T lymphocytes (CTL) that are detected in the spleens of inbred mice after infection with PV.

Primary i.v. inoculation of mice with PV resulted in augmented spleen NK activity that peaked at 3- 4 days after infection. This NK response was followed by an H-2 restricted, virus-specific CTL response that peaked 3 days later. Rechallenge of PV-primed mice with homologous virus resulted in a slight but significant increase in spleen NK activity 1 day after reinfection, and this was followed 3 days later by peak CTL activity. Thus, memory cell-mediated immune responses appeared more rapidly after secondary in vivo challenge with PV. Furthermore, the temporal kinetic relationship between virus-induced NK and CTL responses was maintained after both primary and secondary infection with PV, which suggested that virus-induced NK cells may represent pre-CTL.

To investigate the relationship between these two cytotoxic cell populations, expression of lineage specific cell-surface antigens on virus-induced NK and CTL effectors was examined. NK cells induced after primary and secondary infection with PV were found to rapidly acquire the pan-T cell marker Thy-1, which was expressed on mature anti-viral CTL. In addition, asialo-GM 1 (a glycolipid which

has been considered a marker of NK cells) appears to be expressed on PV-specific CTLp; treatment of PV-primed spleen cells with a polyclonal rabbit antiserum to this marker plus complement prior to secondary in vitro restimulation with PV-infected macrophages prevented the generation of secondary CTL responses to PV. Furthermore, multiple i.v. injections of this antiserum were able to abrogate the in vivo generation of both NK and CTL responses after primary or secondary infection with PV.

Secondary NK and CTL responses were generated in mice that had been pretreated with cyclophosphamide (CY), suggesting that memory cell-mediated immune responses can be reactivated in vivo without undergoing cell division. In contrast, treatment with CY before primary infection delayed the appearance of virus-induced NK activity and abrogated the generation of H-2 restricted, virus-specific CTL. Rechallenge of these CY-treated, NK-primed mice resulted in the rapid generation of a secondary NK response that was not followed by either a primary or secondary CTL response. This long-term block in CTL generation was not due to the establishment of a persistent PV infection. Memory CTL generation could be restored by secondarily coinfecting mice with PV and a second arenavirus such as Tacaribe virus (TV) or lymphocytic choriomeningitis virus (LCMV), or by injection of interleukin 2 (IL 2)-containing supernatants after rechallenge with PV. To demonstrate that IL 2 was the responsible lymphokine in these supernatants, highly purified IL 2 was added to in vitro cultures of spleen cells from CY-treated, PV-primed mice. In the presence of PV-infected syngeneic macrophages, addition of purified IL 2 resulted in a dose dependent restoration of H-2

restricted, anti-PV CTL activity. In addition, the CTL precursor frequency of CY-treated, PV-primed mice appeared to be markedly reduced compared to that in normal PV-primed mice. Thus, the long-lasting block in the ability to generate PV-specific memory CTL appears to be due to both a lack of helper T cell activity and a significant reduction in the number of CTLp. Furthermore, these results suggest that priming the NK compartment is sufficient to prime for a memory CTL response, provided helper factors such as IL 2 are supplied.

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This thesis is dedicated to my mother, Juanita,

and my father, Scott.

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AK	anomalous killer
AsGM 1	gangliotetraosylceramide (asialo GM 1)
C'	complement
Con A	concanavalin A, a lectin
CTL	cytotoxic T (thymus derived) lymphocyte
CTLp	cytotoxic T lymphocyte precursor
CY	cyclophosphamide
DTH	delayed type hypersensitivity
FcR	low affinity Fc receptor for IgG
FCS	fetal calf serum
GM 1	monoganglioside GM 1
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanosulfonic acid
HSV	Herpes Simplex virus
i.c.	intracranial
IFN	interferon
IgG	immunoglobulin G
IL 2	interleukin 2 (T cell growth factor)
i.p.	intraperitoneal
i.v.	intravenous
LCMV	lymphocytic choriomeningitis virus
LGL	large granular lymphocyte
MEM	minimal essential medium
MHC	major histocompatibility complex
MLC	mixed lymphocyte reaction
MOI	multiplicity of infection (number of pfu/cell)
MV	Munchique virus
NK	natural killer
NKTa	clonotypic receptor on NK cells for antigen recognition
PBL	peripheral blood leukocyte
PBS	phosphate buffered saline
pfu	plaque forming unit
PV	Pichinde virus
rIL 2	IL 2 produced by recombinant DNA technology
SSPE	subacute sclerosing panencephalitis
TcR	MHC-restricted T cell receptor for antigen
TCDF	T cell differentiation factor
Ti	clonotypic receptor for antigen plus MHC on T cells
TV	Tacaribe virus
VSV	vesicular stomatitis virus

CHAPTER ONE

INTRODUCTION

A number of chronic human diseases such as progressive rubella panencephalitis, subacute sclerosing panencephalitis, and Kuru are caused by persistent viral infections, and a viral etiology is suspected in others (Wolinsky and Johnson, 1980; Stroop and Baringer, 1982). In addition, persistence of Hepatitis B virus and the AIDS-associated retrovirus constitute major public health problems of world-wide concern (Vyas and Blum, 1984; Fauci, 1985). The host and viral factors that are involved in the establishment and maintenance of persistent infections are not well defined.

The arenaviruses are a family of enveloped viruses that cause acute and persistent infections of rodents in nature (reviewed by Howard and Simpson, 1981; Lehmann-Grube, 1984). Four arenaviruses, notably Lassa virus, Junin virus, Machupo virus, and lymphocytic choriomeningitis virus (LCMV), are capable of causing severe disease in humans. LCMV, the prototype arenavirus, is maintained in nature as a persistent infection in mice. Experimental infection of mice with LCMV produces diverse disease syndromes (depending on the strain and dose of virus, the route of infection, and the strain of mouse), and thus is an excellent model system for defining the conditions that lead to viral clearance or persistence (reviewed by Lehmann-Grube, 1982; Lehmann-Grube et al., 1982a).

Infection of adult mice with LCMV produces a characteristic disease (lymphocytic choriomeningitis) which terminates in death or leads to recovery with elimination of the virus. Clearance of the virus is mediated by virus-specific cytotoxic T lymphocytes (CTL), which lyse histocompatible LCMV infected target cells in vitro with

great efficiency (Zinkernagel and Doherty, 1974a; Zinkernagel and Welsh, 1976; Byrne and Oldstone, 1984). This T cell response, which peaks 7- 10 days after primary infection, also causes the characteristic central nervous system disease of LCM; virus-specific CTL apparently attack LCMV-infected cells in the choroid plexus and meninges (Cole and Nathanson, 1974). Infection of newborn mice with LCMV results in persistent infection which remains clinically inapparent, although high titers of virus are present throughout life in all organs. Numerous attempts to demonstrate a CTL response in these carrier mice have failed (Marker and Volkert, 1973; Cihak and Lehmann-Grube, 1974; Zinkernagel and Doherty, 1974b). Suppression of CTL activity is probably important for the maintenance of the carrier state, as adoptive transfer of Thy-1 positive spleen cells from LCMV-immune mice into persistently infected mice leads to clearance of the virus (Volkert et al., 1974).

Since the CTL response to Pichinde virus (PV; a related arenavirus) is normal in LCMV carrier mice, the mechanism for suppression must be highly specific for LCMV-reactive T cells (Buchmeier et al., 1980). The absence of LCMV-reactive T lymphocytes in persistently infected mice is currently explained either by clonal deletion (Dunlop and Blanden, 1977; Cihak and Lehmann-Grube, 1978) or clonal suppression (Zinkernagel and Doherty, 1974b). All attempts to demonstrate the presence of suppressor cells have been unsuccessful (Cihak and Lehmann-Grube, 1978), and thus persistence of LCMV may be related to its ability to infect lymphoid cells (Borden and Nathanson, 1974). Selective infection of LCMV-specific T cells (perhaps via their immunological receptors) could account for the abrogation of CTL

activity (Popescu et al., 1979). The virus could be directly cytolytic for LCMV-specific T cells, leading to clonal deletion of virus-specific CTL, or infection could result in functional inactivation by inhibiting antigen recognition and/or lytic mechanisms. Indeed, recent studies by Ahmed and coworkers (1984) indicate that life-long suppression of T cell responses in adult LCMV carrier mice could be caused by genetic variants of LCMV that infect and inactivate mature T cells, thus contributing to the persistence of the virus. An alternate hypothesis was recently proposed by Lehmann-Grube, after it was demonstrated that T cells from newborn mice could be infected with LCMV, whereas resting or activated T cells from adult mice could not (Popescu et al., 1982; Lehmann-Grube et al., 1983). Based on these observations it was postulated that the virus infects T cells at an early stage of development and blocks their differentiation into mature CTL (Lehmann-Grube et al., 1983). Mature CTL in the adult mouse would be resistant to infection and capable of responding to challenge with the virus. This hypothesis is consistent with the observation that an inverse relationship exists between the age at which the mouse is infected with LCMV and survival with virus persistence (Lehmann-Grube et al., 1982a). Thus, the outcome of LCMV infection (i.e. persistence or acute infection) may be related to the ability of the virus to recognize and replicate in a specific subset of lymphoid cells.

Variation in the outcome of PV infection in two strains of Syrian hamster also appears to be determined by the ability of the virus to infect a subset of lymphoid cells in the host's spleen. Buchmeier and Rawls (1977) found that PV caused a fatal infection in

adult inbred MHA hamsters but not LSH hamsters after i.p. infection. The lethal infection was accompanied by high titers of virus in the spleen, but this could not be attributed to an inability of MHA strain hamsters to mount an immune response to viral antigens. Rather, the susceptible (MHA) strain appeared to have greater numbers of splenic target cells capable of replicating the virus to high titers. Genetic analysis of the susceptible and resistant hamsters revealed that susceptibility is controlled by a single autosomal recessive gene, and this was correlated with enhanced natural killer (NK) cell activity in the spleens of susceptible animals (Gee et al., 1979; 1981a). In addition, virus-producing cells in the spleen co-purified with lymphocytes having NK cell activity (Gee et al., 1981b). Taken together, these results suggest that the target cell for virus replication in the initial phase of infection is a spleen cell with NK activity. A hypothesis has recently been developed to account for differences between LSH and MHA hamsters (Wright et al., 1984). It was proposed that the target cell for virus replication is a precursor T lymphocyte that has spontaneous NK activity in the spleen. Exposure to virus triggers the differentiation of the precursor to the mature T cell stage, which is not permissive for viral replication. According to this hypothesis, T cell maturation occurs normally in resistant (LSH) hamsters, but susceptible (MHA) hamsters possess a block in the maturation pathway that results in accumulation of precursor (NK) cells that are permissive for virus replication. Another possibility is that the differentiation pathway is normal in MHA hamsters but a defect in lymphopoeisis results in overproduction of precursor cells.

One prediction of this hypothesis is that cells with NK

activity in the hamster reside in the T lineage. However, recent studies in the human and murine systems have revealed that NK cells express differentiation antigens associated with the monocyte/macrophage as well as the T cell lineages. This heterogeneity has complicated attempts to identify the lineage to which NK cells belong; a relationship to T cell (Herberman et al., 1979), monocyte (Lohmann-Mathes et al., 1979), and independent lineages (Ortaldo, 1982) have been proposed. Alternatively, NK cell activity may be mediated by a composite of cell types, with some deriving from the T lineage while others derive from the monocyte lineage (Ortaldo and Herberman, 1984).

1.1 Characteristics of NK cells and their relationship to the T lineage

1.1.1 Definition of cytotoxic cell populations

Natural killer cells are defined operationally as a sub-population of lymphocytes that are capable of lysing a variety of normal, neoplastic, and virus-infected target cells in the absence of prior sensitization (Ortaldo and Herberman, 1984; Trinchieri and Perussia, 1984). In contrast, the cytotoxic activity of CTL is antigen-specific, and the generation of a CTL response requires in vivo and/or in vitro sensitization (reviewed by Zinkernagel and Doherty, 1979). NK cells also differ from classical CTL in that they do not recognize antigen in association with cell surface molecules encoded by the major histocompatibility complex (MHC), a phenomenon termed MHC restriction.

A novel type of cytotoxic cell response is generated when human peripheral blood leukocytes (PBL) or mouse splenocytes are

co-cultured with allogeneic stimulator cells (Paciucci et al., 1980; Karre and Seeley, 1979, Lopez-Botet et al., 1982), tumor cells (Shain, 1982), lectin (Hunninghake and Fauci, 1977), lymphokine-conditioned medium (Teh and Yu, 1983; Kasakura, 1977), or purified IL 2 (e.g. Rosenberg et al., 1984- discussed below). This culture-induced cytotoxicity is termed "anomalous" or "NK-like" as the relationship of the effector cells to classical NK cells or CTL is not clear (Koren and Herberman, 1983; reviewed by Ortaldo and Herberman, 1984). Anomalous killer (AK) cells display a number of T cell differentiation antigens that are not expressed on freshly isolated NK cells, and the target cell specificity of AK and NK effectors is different. AK cells will kill YAC-1 and K562 cells (traditionally used as susceptible targets for murine and human NK, respectively), as well as a broad range of NK insensitive tumor and virus-infected cells. Since cytotoxicity mediated by AK cells is not MHC restricted, they can also be distinguished from CTL.

Cytotoxic cells with NK-like activity are also induced in mice and humans after infection with a variety of viruses. In the mouse system, this virus-activated natural killer cell response peaks early after infection prior to the appearance of MHC restricted CTL (reviewed by Welsh, 1981). It has been postulated that virus-activated natural killer cells are the in vivo counterpart of in vitro generated AK cells, as both effector populations share the same broad target cell repertoire and T cell-like antigenic phenotype (Klein et al., 1980a; 1980b).

The ontogenetic relationship between NK, AK, and CTL effectors is not clear, but it has been hypothesized that all three

cytotoxic activities are mediated by largely overlapping subsets of effector cells that reside in the T lineage (Klein, 1980a; 1984; Grossman and Herberman, 1982). This contention is supported by the observation that NK and AK effectors share certain phenotypic and functional characteristics with CTL, including morphology, expression of certain cell surface markers, and cytolytic mechanisms. Thus, non-specific cytotoxic activity may be an additional property of antigen-specific CTL that are at different stages of activation and/or differentiation.

1.1.2 Morphology and origin of NK cells

Most NK activity in the human peripheral blood and rodent spleen is mediated by large lymphocytes with distinctive azurophilic granules in the cytoplasm (the so-called LGL or large granular lymphocytes; Timonen et al., 1981; Reynolds et al., 1981; Kumagi et al., 1982). This distinctive morphology has been considered a marker for NK cells, but it appears that antigen-specific CTL can also have cytoplasmic granules that are indistinguishable from those of NK cells at the ultrastructural level (Grossi et al., 1983). Thus, the presence of granules may be a morphological marker for all cytolytic lymphocytes, regardless of antigen specificity. In the human and rat, LGL comprise only 5% of peripheral blood or spleen leucocytes, respectively (Timonen et al., 1981; Reynolds et al., 1981). Relatively pure populations of LGL can be isolated on Percoll density gradients, and these have been widely used in studies of NK cell phenotype and function.

Mature NK cells appear to originate from non-cytolytic precursor cells that mature in the bone marrow (Haller et al., 1977;

Hackett et al., 1985). The bone marrow also appears to be one site of NK cell maturation, as mice treated with ^{89}strontium or beta-estradiol (both of which destroy the bone marrow microenvironment) fail to generate NK activity when reconstituted with syngeneic bone marrow cells (Seaman and Talal, 1980; Kumar et al., 1979). An intact thymus, which is required for optimal CTL development, is not necessary for the differentiation of NK cells, as high levels of NK activity have been detected in athymic nude mice or in neonatally thymectomized mice (Ortaldo and Herberman, 1984).

1.1.3 Regulation of NK cell activity

Expression of cytolytic activity by NK, AK, and CTL effectors is controlled by a variety of soluble mediators, among which IL 2 and the interferons (IFN) probably play a major role (reviewed by Trinchieri and Perussia, 1984; Ortaldo and Herberman, 1984; Farrar et al., 1982). The existence of common regulatory molecules and pathways supports the contention that a close relationship exists between all three subpopulations of cytotoxic lymphocytes.

All three types of IFN (alpha, beta, and gamma) are able to enhance the NK activity of human PBL or mouse splenocytes in vitro (Trinchieri et al., 1978; Djeu et al., 1979; Senik et al., 1980; Svedersky et al., 1984). IFN boosts cytotoxicity by converting non-cytolytic precursors into active NK cells and by increasing the efficiency of killing by pre-existing NK cells (Minato et al., 1981; Targan and Dorey, 1980; Timonen et al., 1982b). In addition, IFN induces resting NK cells to undergo blastogenesis and cell division in vivo, which suggests that IFN-mediated boosting of non-specific cytotoxicity is due to proliferation as well as activation of NK cells

(Santoni et al.,1985). Virus-activated NK activity detected in the mouse spleen after infection with LCMV also appears to be mediated by large blast-like cells that are dividing (Biron and Welsh, 1982). It has been postulated that IFN produced in response to virus infection is responsible for activation and proliferation of NK cells, as a strong correlation has been observed between serum IFN levels and enhanced NK activity after virus infection (Welsh, 1978). This view is further supported by the observation that in vivo administration of anti-IFN antibodies abrogated Newcastle disease virus-induced NK activity (Gidlund et al., 1978). However, it is also possible that augmented NK cell activity is triggered by direct exposure to viral glycoproteins; in vitro stimulation of human PBL with purified measles, LCMV, or mumps virus glycoproteins resulted in an enhanced NK cell response within 4 hrs of treatment, and IFN could not be detected in these systems (Casali et al., 1981; Harfast et al., 1980). The role of virus- induced IL 2 in the boosting of NK activity in vivo is also not clear.

IL 2 is a soluble regulatory molecule that is required for the activation and proliferation of NK, AK, and CTL effectors. Resting T lymphocytes express high affinity receptors for IL 2 after stimulation with mitogen or antigen (Larsson et al., 1980; Robb et al., 1981) and thus become receptive to the proliferative signal of IL 2 (Smith, 1984), which is produced predominately by cells of the T helper subset. The generation of functional CTL from non-cytolytic precursors (CTLp) appears to require 2 signals; IL 2 provides the signal for CTLp to proliferate while a second lymphokine (possibly distinct from IL 2) induces differentiation of the precursor cells

(Raulet and Bevan, 1982; Wagner et al., 1982; Mannel et al., 1983; Kanagawa, 1983; Finke et al., 1983; Garman and Fan, 1983; Hardt et al., 1985).

Human and murine NK cells respond directly to IL 2 with enhanced cytotoxic activity and eventually proliferation, with no additional requirement for activating stimuli such as antigen or mitogen. Purified human LGL display enhanced NK activity within 1- 4 hrs of treatment with IL 2 produced by recombinant DNA technology (rIL 2; Svedersky et al., 1984; Trinchieri et al., 1984; Ortaldo et al., 1984; Lanier et al., 1985; Kabelitz et al., 1985), by a process that requires protein but not DNA synthesis (Lanier et al., 1985). In addition to enhancing NK activity in short term cultures, rIL 2 can induce, in in vitro cultures of 3 days or more, cells with NK-like activity that are cytotoxic for NK-insensitive target cells (Rosenberg et al., 1984; Merluzzi et al., 1984a; Devos et al., 1984; Ortaldo et al., 1984; Itoh et al., 1985a). Mediators of AK activity are large blast cells and the response requires cell division. An AK response can also be generated in vivo by injecting mice with rIL 2; the effector cells are similar to in vitro generated AK cells in that they express T-cell differentiation antigens and kill NK-sensitive and insensitive target cells (Ettinghausen et al.,1985).

The proliferative response of NK cells to IL 2 has provided the means for establishing cloned, IL 2-dependent NK cell lines, which have been useful in defining subsets of NK cells that differ in cell surface phenotype and antigen-specificity (discussed below). Long-term growth of NK cell lines in vitro requires (or is enhanced by) humoral or cellular factors in addition to IL 2, but they remain

poorly defined (Olabuenaga et al., 1983; Roberts and Moore, 1985).

Stimulation of T lymphocytes or NK cells with IL 2 induces the production of gamma-IFN (Kasahara et al., 1983a; Handa et al., 1983; Trinchieri et al., 1984), which may be required for the activation of NK, AK, and CTL effectors. A role for this lymphokine in the induction of killer cell responses is supported by the observation that production of gamma-IFN precedes the generation of AK or CTL activity in vitro (Farrar et al., 1981; Weigent et al., 1983; Itoh et al., 1985b). Further, addition of polyclonal or monoclonal anti-gamma IFN antibodies to culture supernatants blocks the generation of AK and CTL responses in vitro (Farrar et al., 1981; Weigent et al., 1983; Itoh et al., 1985b), and partially reduces IL 2 induced enhancement of NK activity (Ortaldo et al., 1984). It has been postulated that gamma-IFN acts as a differentiation factor, perhaps by increasing the number of IL 2 receptors on cytotoxic cell precursors (Johnson and Farrar, 1983; Itoh et al., 1985b).

Thymulin (formerly designated serum thymic factor or FTS) is a hormone produced by thymic epithelial cells that regulates T-cell dependent immune responses and plays an important role in T cell differentiation (Bach, 1983; Palacios, 1983). Recent studies have shown that thymulin also modulates the activity of murine NK cells after in vivo (Bardos and Bach, 1982) or in vitro (Kaiserlian et al., 1983) treatment; it can either enhance or suppress lytic activity depending on the genetic background and age of the mouse. Other thymic hormones, including thymosin and thymopoietin (Stutman, 1983; Goldstein et al., 1983; Zatz et al., 1985), also affect T cell and NK cell responses (Kaiserlian et al., 1983; Herberman et al., 1979).

Regulation of NK activity by these thymic factors argues in favour of a close relationship to the T-cell lineage.

1.1.4 NK cell surface antigens

The relationship between NK cells and other leukocyte subpopulations has been investigated using antibodies that react with lineage-specific cell surface differentiation antigens. Early serological studies revealed that the cytotoxic activity of human or murine NK cells is sensitive to treatment with anti-T cell serum and complement, and thus it was concluded that they reside in the T lineage (Kaplan and Callewaert, 1978; Herberman et al., 1978). However, the finding that not all NK cells express T cell markers, and that some share antigens with myelomonocytic cells, has reopened the question of NK cell lineage.

Antigens expressed preferentially on NK cells. Attempts to identify surface antigens on NK cells which are unique or characteristic of all cells with this functional activity have not been successful. Rather, NK cells display a unique combination of antigens, each shared with other cell types including T lymphocytes or monocytes. Virtually all human NK cells bear a low affinity Fc receptor (FcR) for immunoglobulin G (IgG), which is also strongly expressed on polymorphonuclear leukocytes (PMN) (Jondal and Pross, 1975; Fleit et al., 1982). Three monoclonal antibodies (designated Leu-11, 3G8, and B73.1) have been raised against this marker; B73.1 recognizes an epitope that is expressed preferentially on NK cells whereas 3G8 and Leu-11 recognize one or more epitopes on both NK cells and PMN (Perussia and Trinchieri, 1984; Perussia et al., 1984; Phillips and Babcock, 1983). The majority of B73.1 positive cells have the LGL

almost all spontaneous cytotoxic activity is recovered in this subset after immunoaffinity purification (Perussia et al., 1982; 1983). Further, the B73.1 positive population is completely non-overlapping with B and T cells, as defined by specific surface antigens (Perussia et al., 1982; 1984; Perussia and Trinchieri, 1984).

The reactivity of monoclonal antibody Leu-7 (HNK-1), which was originally described as NK specific, is complex (Abo and Blach, 1981). In addition to recognizing a variable portion (20- 60%) of cytotoxic NK cells in the human peripheral blood (Perussia et al.,1984; Lanier et al., 1983; 1984), Leu-7 antibodies also detect a subset of non-cytolytic lymphocytes in various lymphoid tissues that have been postulated to be pre-NK cells (Abo et al., 1982; 1983a; 1984). However, it is now clear that anti-Leu-7 will also react with freshly isolated and cultured T cells (Abo and Blach, 1981; Abo et al., 1983b), pre-B cell lines (Findley et al., 1982), and myelin-associated glycoprotein (McGarry et al., 1983). Thus, it has been suggested that Leu-7 is a differentiation antigen that is expressed on a variety of hemopoietic and non-hemopoietic cell types.

Hercend and co-workers (1985) have recently developed a pan-NK cell monoclonal antibody designated NKH1 that reacts with the entire pool of NK active lymphocytes and appears to define a cell surface antigen that is distinct from B73.1 or Leu-7. Preliminary screening has revealed that it does not recognize any other hemopoietic cell types in the peripheral blood (including mature T and B lymphocytes, monocytes and granulocytes), but its expression on T cells at early stages of differentiation (such as thymocytes and pre-thymocytes) has not been examined (Griffin et al., 1983; Hercend

et al., 1985).

The murine alloantigens NK-1 and NK-2, which are currently recognized as the only NK-specific markers, are expressed on the majority of splenic NK cells and on immature precursor NK cells in the bone marrow (Glimcher et al., 1977; Pollack et al., 1979; Burton and Winn, 1981; Koo et al., 1984). The observation that polyclonal antisera to NK-1 and NK-2 react with some cloned T cell lines is consistent with the view that there is an association of NK cells with the T lineage (Brooks et al., 1983a). NK cells also express the neutral glycolipid asialo-GM1 (asGM1) which has been considered a marker for murine NK cells (Young et al., 1980; Kasai et al., 1980a). Splenic NK activity is eliminated when mice are injected with a rabbit antiserum to asGM1 (Kasai et al., 1980b; Kawase et al., 1982), or when the effector cells are treated in vitro with the anti-serum plus complement (Young et al., 1980; Kasai et al., 1980a).

NK cells activated by virus infection in vivo or lymphokine conditioned medium in vitro are less sensitive to treatment with anti-NK-1 or anti-asGM1 antibodies and complement (Kumar et al., 1982; Karre et al., 1983; Yang et al., 1985), but they do display increased amounts of certain T cell differentiation antigens (discussed below), which is consistent with the view that they are maturing to become functional T cells after exposure to antigen and/or growth factors. Similarly, human LGL appear to lose certain non-T cell markers (i.e. B73.1 and Leu-7) after activation in vitro, while levels of T-cell associated antigens are increased (Timonen et al., 1982; Burns et al., 1984; Roberts and Moore, 1985).

Myelomonocytic differentiation antigens on NK cells. The finding that

monoclonal antibodies OKM 1 and MAC 1 react with both NK cells and myelomonocytic cells in the human and mouse has raised the possibility of a relationship to the myeloid lineage (Kay and Horowitz, 1980; Ault and Springer, 1981; Beller et al., 1982). However, the antigen recognized by these antibodies (the C3Bi complement receptor) is not expressed exclusively on monocytes; OKM 1, for instance, also reacts with PMN and a sub-population of T cells that bear the FcIgG receptor (the so-called T-gamma subset) (Ortaldo et al., 1981; van de Griend et al., 1982). Thus, it has been proposed that these antigens are not myelomonocytic-restricted markers, but are differentiation antigens shared by cells of various lineages (Ortaldo and Herberman, 1984). The finding that monoclonal antibodies with more selective reactivity against human monocytes (i.e. MO2) do not recognize human LGL also argues against the hypothesis that NK cells reside in the myeloid lineage (Todd et al., 1983).

T cell antigens on NK cells. The T cell antigen most widely expressed on human NK cells is OKT11, the low affinity receptor for sheep erythrocytes. Monoclonal antibodies to this marker react with 80- 90% of freshly isolated NK cells from the peripheral blood as well as all mature T lymphocytes (Perussia et al., 1982; Zarling et al., 1981). It has been proposed that OKT 11 is an antigen-independent activation pathway for T cells and NK cells as anti-OKT 11 antibodies can induce expression of high affinity IL 2 receptors on both cytotoxic cell populations, which leads to subsequent proliferation as long as IL 2 is present (Meuer et al., 1984a; Schmidt et al., 1985). Presumably, a natural ligand that has yet to be identified can also bind to the OKT 11 surface antigen and activate cytotoxic cells in the absence of

antigen. Most human NK cells also react with the OKT 10 monoclonal antibody, which detects a 46,000 mw protein on thymocytes and activated T cells, but not quiescent peripheral blood T cells (Ortaldo et al., 1981). It is not known if murine NK and T lymphocytes share surface antigens equivalent to human OKT 10 and OKT 11 surface markers.

A number of T-lineage restricted surface glycoproteins that play a role in target cell recognition by T cells have been defined by monoclonal antibodies. All human T cells express T3, a 20 kD glycoprotein that appears late in intrathymic ontogeny at about the time cells become immunocompetent (Kung et al., 1979; Reinherz et al., 1980a; 1980b; Umiel et al., 1982). It has been proposed that the murine Thy-1 surface marker is the structural and functional counterpart of the human T3 antigen, because both are expressed on all mature mouse and human T lymphocytes, respectively (Gunter et al., 1984; Reif and Allan, 1964; Raff et al., 1969; Reinherz et al., 1980a). However, unlike anti-T3 antibodies, anti-Thy-1 does not block functional responses by murine T cells (Gunter et al., 1984; Reinherz et al., 1980). Further, T3 molecules form complexes with antigen-recognition structures on the surface of human T cells (Meuer et al., 1983a; Brenner et al., 1985), but Thy-1 does not associate with the murine T cell receptor for antigen (Gunter et al., 1984). A homolog of T3 (called L4T3) that is distinct from Thy 1 has recently been described on murine T cells (Allison et al., 1985; van den Elsen et al., 1985). It has a similar structure to T3 (van den Elsen et al., 1985), is non-covalently associated with the murine T-cell antigen receptor, and antibodies directed against it block T cells from

responding to antigen (Allison et al., 1985).

The expression of these pan T cell markers on human and murine NK cells has been examined. The NK activity mediated by freshly isolated human PBL is not sensitive to treatment with anti-T3 antibodies and complement (Zarling and Kung, 1980), but T3-negative PBL that have been enriched for NK activity (either on Percoll gradients or by immunoaffinity procedures with monoclonal antibody B73.1) will display the T3 antigen after in vitro activation with lymphokine-conditioned medium or allogeneic stimulator cells (Grimm et al., 1983; Burns et al., 1984; Roberts and Moore, 1985). Some cloned NK cell lines may also express the T3 marker (Sheehy et al., 1982; Pawelec et al., 1982a; Allevena and Ortaldo, 1984; Hercend et al., 1983a), and monoclonal anti-T3 antibodies will block non-specific cytotoxic activity by these effector cells (Ritz et al., 1985). Expression of the murine homolog of T3, L3T4, on mouse NK cells has not been investigated. Most freshly isolated splenic NK cells do not express Thy-1 (or express it at low levels) (Herberman et al., 1977; Pollack et al., 1979; Koo et al., 1980), but virtually all AK effectors and NK cell clones express this antigen after in vitro culture (Hengartner et al., 1982; Karre et al., 1983; Dennert et al., 1981; Brooks et al., 1982). Non-specific killer cells detected in vivo after exposure to virus also appear to acquire the Thy-1 antigen as the infection progresses (Herberman et al., 1977; Kiessling et al., 1980; Walker et al., 1984). Thus, it appears that pan T cell markers are absent from freshly isolated mouse or human NK cells, but activation with antigen and/or growth factors induces their expression.

Two antigenically distinct sub-populations of T-lymphocytes have been described in both humans and mice. In the human system these subsets are defined on the basis of their unique 62 kD and 76 kD membrane markers, termed T4 and T8, respectively (reviewed by Reinherz and Schlossman, 1980). This segregation is a late event in intrathymic development, as immature thymocytes express both T4 and T8 on their surface (Reinherz et al., 1980b). The murine analogs of T4 and T8 are the L3T4 and Lyt 2 surface antigens, respectively (Dialynas et al., 1983; Cantor and Boyse, 1975). Traditionally, these differentiation antigens have been considered markers of T cell function, with cytotoxic/suppressor cells expressing T8 (Lyt 2 in the mouse) and helper/inducer cells expressing the T4 antigen (L3T4 in the mouse)(Reinherz and Schlossman, 1980; Dialynas et al., 1983; Cantor and Boyse, 1975). However, it is now clear that a small percentage of CTL are T4 positive (Engleman et al., 1981; Spitz et al., 1982; Krensky et al., 1982; Meuer et al.,1982; Moretta, 1985). A more stringent relationship exists between T4 and T8 cells and the histocompatibility proteins on the target cell with which they interact; T4 positive cells recognize antigen in association with class II MHC gene products (HLA-DP,DO,DR), while T8 positive cells interact with class I MHC antigens (HLA-A,B,C) on the target cell surface (Meuer et al., 1982; Engelman et al., 1981; Krensky et al., 1982). A similar situation exists in the mouse, where expression of L3T4 or Lyt 2 correlates not so much with function as with recognition by T cells of class II (H-2I) or class I (H-2 K,D,L) MHC antigens, respectively (Swain, 1981, 1983a; Dialynas et al., 1983; Lukacher et al, 1985). The function of these T cell subset markers is not clear,

but it has been suggested that they serve to increase the avidity of, or stabilize the interaction between, the T cell and its target (Meuer et al., 1982; MacDonald et al., 1982; Marrack et al., 1983; Moretta, 1984a; Biddison, 1984), perhaps by binding to non-polymorphic regions of the appropriate MHC surface molecule (Biddison et al., 1982; Swain, 1983b).

The expression of T cell subset markers on freshly isolated and/or cultured NK cells suggests some relationship to the T lineage. The OKT 4 surface antigen (which is displayed predominately on T helper lymphocytes) is not found on freshly isolated human NK cells (Ortaldo et al., 1981), but it can be detected on some long-term NK cell lines (Moretta et al., 1981; Sugamura et al., 1982; Allavena and Ortaldo, 1984). Expression of L3T4 (the functional counterpart of OKT4 in the mouse) on murine NK cells has not been examined. However, monoclonal antibodies to Lyt 1 , which is also a marker of the T helper subset, react with about 25% of murine splenic NK cells (Koo et al., 1980). A subset of human NK cells, AK effectors, and long-term NK cell lines express the OKT8 antigen (Grimm et al., 1983; Burns et al., 1984; Perussia et al., 1983b; Zagury et al., 1985). Lyt 2 (the murine homolog of OKT8) is not expressed on NK cells in the murine spleen, but it can be detected on cultured AK cells and continuously growing NK cell lines (Shain et al., 1982; Brooks et al., 1982; Shortman et al., 1983).

The development of functional, antigen-specific T cell clones and clone-specific monoclonal antibodies has permitted the characterization of the antigen receptor on MHC restricted T cells in mice (Haskins et al., 1984) and humans (Meuer et al., 1984b). In both

species the receptor is a 90 kD heterodimer (designated Ti) that is composed of disulphide linked alpha and beta chains, each of which has a mw of 40- 45 kD (Meuer et al., 1983b; Haskins et al., 1983). Peptide mapping studies have revealed that each of the alpha and beta subunits seem to contain constant and variable regions (Acuto et al., 1983; Kappler et al., 1983).

The genes encoding these proteins have been cloned and shown to resemble immunoglobulin in overall structure and the requirement for DNA rearrangement prior to expression (reviewed by Hood et al., 1985). Comparison of alpha and beta chain cDNA with corresponding genomic or germline DNA has revealed variable (V), diversity (D), joining (J), and constant (C) gene segments that must be rearranged into a complete transcriptional unit before gene expression can occur (Hedrick et al., 1984; Yanagi et al., 1984; Chien et al., 1984; Saito et al., 1984a). A third receptor-like gene, designated gamma, has also been described (Saito et al., 1984b). It is similar to the alpha and beta genes in that it has V, J, and C gene segments, and it is rearranged and expressed in T cells but not B cells (Saito et al.,1984b). However, the gamma gene does not encode any of the T cell receptor (TcR) components identified by numerous monoclonal antibodies, and its function has not yet been determined.

Molecular analysis of immunoglobulin gene rearrangement and expression has been useful in defining the differentiation pathway of B cells. Since rearrangements represent a specific and early event in B cell differentiation they provide an irreversible marker for committment to the B cell lineage (reviewed by Wall and Kuehl, 1983; Calame, 1985). In addition, rearrangement events occur in an ordered

fashion during B cell development; heavy chain rearrangement occurs before light chain rearrangment, with kappa light chain preceding the lambda light chain (Wall and Kuehl, 1983; Calame, 1985). Thus, depending on the pattern of Ig gene rearrangements in a clonal B cell population, one can determine its stage of maturation. Similarly, rearrangment of the alpha and beta TcR genes is restricted to cells of the T lineage, and thus rearrangement could be used as a marker to determine the relationship between NK and T lymphocytes. Further, since rearrangement of TcR genes also appears to occur in a hierarchical pattern during T cell ontogeny (i.e. the beta chain gene is rearranged before the alpha gene), it may also be possible to identify where NK cells fit into the T cell differentiation pathway (Raulet et al., 1985; Collins et al., 1985; Snodgrass et al., 1985). Recently, Yanagi and co-workers (1985) demonstrated that the TcR beta chain gene is rearranged and expressed in cloned murine NK cell lines and freshly isolated NK cells from the spleens of mice treated with Poly I:C (an interferon inducer). Similarly, it has been shown that some human NK cell clones have a rearranged or partially rearranged beta chain gene (Ritz et al., 1985). The alpha chain gene was rearranged and expressed only in human NK clones that displayed the pan T cell marker T3, and the gamma chain gene was not trahscribed in any of the clones (Ritz et al., 1985).

It has been postulated that the proteins encoded by the TcR genes are involved in recognition of antigen by NK cells (Yanagi et al., 1985). The observed correlation between NK activity and the level of TcR beta chain mRNA expressed in murine NK cell clones supports this hypothesis (Yanagi et al., 1985). In the human,

monoclonal antibodies have been raised against a 90 kD heterodimer on the surface of two NK cell clones (Hercend et al., 1983b). This structure, termed NKTa, is linked to the T3 molecule on the surface of the clones, appears to be the product of functionally rearranged T cell receptor genes, and is intimately involved in determining the target specificity of the two NK clones against which the clonotypic antibodies were raised (Hercend et al., 1983b). However, not all of the clones used in that study expressed a T3-NKTa complex. Further, Reynolds and co-workers (1985) have found that the beta chain gene is not rearranged and expressed in rat hybridoma lines that have the LGL morphology and NK activity. Taken together, these studies suggest that a subset of NK cells have antigen recognition structures that are analogous to the Ti clonotypic determinants that have been identified on antigen-specific CTL.

The lymphocyte function antigen (LFA-1) is widely distributed on T cells, B cells, and myeloid cells in mice and humans (Sanchez-Madrid et al., 1982; Krensky et al., 1983). Antibodies to LFA-1 can have a variety of effects on T cell function; they are able to block generation of T helper cell responses to antigen, and interfere with conjugate formation between a CTL and its target (Davignon et al., 1981; Sanchez-Madrid et al., 1982). Recent studies indicate that this structure is not part of the antigen receptor on T cells, but rather it appears to be involved in an adhesion strengthening process that stabilizes the bond between the effector cell and its target (Krensky et al., 1984). Human NK cell clones have also been tested for expression of LFA-1 and found to be positive (Schmidt et al., 1985b). Further, anti-LFA-1 antibodies are able to

inhibit non-specific cytotoxic activity by these clones (Schmidt et al., 1985b), and by freshly isolated NK cells from the human peripheral blood (Krensky et al., 1983). This finding, together with other studies showing that NK and T lymphocytes share a common lytic mechanism, lends further support to the view that these two cytotoxic cell populations are closely related (Schmidt et al., 1985b; Henkart, 1985).

In summary, human and murine NK cells display a unique combination of antigens, including some that have been considered markers of the monocyte and T lymphocyte lineages. Thus, in the human most NK cells are OKT3^{-ve}, OKT11^{+ve}, OKT10^{+ve}, OKM 1^{+ve}, B73.1^{+ve}, NKH-1^{+ve}, HNK-1^{+ve}, and LFA-1^{+ve}, while most murine NK cells are Thy-1^{weak}, NK-1^{+ve}, asGM1^{+ve}. Activation of these resting NK cells with antigen (such as viruses) or growth factors (such as IL 2) results in decreased expression of non-T cell markers and a corresponding increase in the amount of pan T cell or T cell subset antigens. This change in NK cell phenotype is consistent with the view that virus-induced NK cells are differentiating to become mature T lymphocytes. This contention is further strenghtened by the finding that NK and T lymphocytes share the LFA-1 surface antigen, which appears to serve a common function on both cytotoxic cell populations.

1.1.5 Specificity of NK cells

The spectrum of target cells susceptible to NK lysis is very broad, but it remains unclear whether one common target structure is recognized by all NK cells, or whether a large repertoire of target antigens can be recognized by discrete subsets of NK cells that have distinct antigen specificities. One approach to this question is to

test the reactivity of NK cells against a panel of tumor targets using cold target inhibition or monolayer absorption techniques; both procedures allow one to identify subsets of NK cells in the rodent spleen or human peripheral blood that react with some NK sensitive tumor targets and not others (Ortaldo and Herberman, 1984). Results from these experiments indicate that at a single cell level most NK cells lyse only one or a few of the array of NK susceptible target cells (Ortaldo et al., 1977; Takasugi et al., 1977; Callawaert et al., 1979; Phillips et al., 1980). Thus, the reactivity of the NK population represents the sum of specifically reactive subsets at the single cell level. This finding has been confirmed using large panels of IL 2-dependent NK cell lines; many of the clones displayed specificities more limited than those recognized by polyclonal NK cell populations (Pawelec et al., 1982c; Kedar et al., 1982; Allavena and Ortaldo, 1984; Zagury et al., 1985). Nevertheless, it has proved difficult to analyze the fine specificity of NK recognition because the tumor cell antigens recognized by NK cells are not well defined.

Natural killer cells have the ability to discriminate between virus-infected and uninfected target cells, and recent studies suggest that recognition may be mediated through viral glycoproteins expressed on the surface of infected cells (Kende et al., 1979; Bishop et al., 1983). Virally encoded surface antigens are well characterized and thus provide a valuable alternative to the use of tumor targets in studies of NK cell recognition. Further, the availability of mutant viruses, and techniques for transfer of individual viral genes into target cells, permits one to manipulate the presence or absence of viral cell surface antigens. In addition, the epitope(s) recognized

by NK effectors can be defined by making discrete changes to the structure of the antigen, or by attempting to block NK reactivity with monoclonal anti-viral antibodies of well defined specificity.

Using these approaches, it has been shown that recognition and lysis of target cells infected with human cytomegalovirus, vesicular stomatitis virus (VSV), and herpes simplex virus I (HSV 1) requires expression of certain viral proteins (Bishop et al., 1984; Borysiewicz et al., 1985; Moller et al., 1985). Conditional lethal mutants of VSV have been used to show that both the G (glycoprotein) and M (matrix) antigens are required for NK-cell mediated lysis of VSV-infected target cells (Moller et al., 1985). However, it was difficult to tell from these studies if NK cells interact directly with the viral proteins, or if they recognized alterations in the cell surface membrane induced by insertion of the G and M molecules. The role of the gB and gC glycoproteins in the recognition of HSV 1 infected target cells by NK effectors has also been examined; monoclonal antibodies to various epitopes on these antigens caused a partial but significant reduction in the NK sensitivity of HSV 1 infected target cells, suggesting that NK cells of different specificities interact directly with viral glycoproteins (Bishop et al., 1983). In addition, since mutations affecting glycoprotein epitopes defined by monoclonal antibodies also influenced the interaction between NK cells and HSV 1 infected targets, it was postulated that viral antigenic sites recognized by NK cells overlap with those recognized by anti-HSV 1 antibodies (Bishop et al., 1984).

Two models have been proposed to account for the observed heterogeneity in target cell recognition by NK cells (Zagury et al.,

1985). First, it is possible that NK cells, like classical T and B lymphocytes, display clonal heterogeneity, with individual clones irreversibly committed to recognizing a particular restricted specificity. In this regard, it has been postulated that certain components of the T cell receptor for antigen play a role in determining NK cell specificity (Yanagi et al., 1985). As noted earlier, this view is supported by the finding that some (but not all) human NK clones express a receptor that is functionally and structurally homologous to the clonotypic antigen receptor on mature T lymphocytes (Hercend et al., 1983). The second possibility is that each NK cell possesses a set of different antigen receptors, only one of which is expressed at a given stage of differentiation. Thus, relatively immature T lymphocytes (i.e. CTLp) would express receptor(s) for NK sensitive targets (such as YAC 1 or K562), whereas mature anti-viral CTL would express a distinct receptor that imparts virus-specificity and H-2 restriction. Such a model would explain the observation that a particular cloned IL 2-dependent T cell line will simultaneously kill both the NK insensitive target against which it was immunized, and an NK sensitive target such as K562 (discussed below).

1.1.6 Induction of NK activity in cloned CTL

A direct approach to the question of NK cell lineage is to study cells of known lineage (i.e. CTL) and determine whether they express NK activity. The specificity of CTL has been studied at the clonal level, either by limiting dilution analysis of precursor cells, or by generating cloned, IL 2-dependent CTL lines.

It has been known for some time that T cell lines can lose

their antigen specific lytic activity while at the same time acquiring NK-like activity (Pawelec et al., 1982b; Hengartner et al., 1982; Lopez-Botet et al., 1983; Shortman et al., 1983; Brooks et al., 1983b; 1983c). These findings have been confirmed and extended by other investigators, who demonstrated that cloned allo-specific or tumor-specific CTL can simultaneously lyse specific or NK-sensitive target cells (Binz et al., 1983; Moretta et al., 1984b; Neefe and Carpenter, 1982). By limiting dilution analysis, Moretta and coworkers (1984) have found that cytotoxic cells can be grouped into 3 categories: those with NK activity alone; those with CTL activity alone; and those that have dual NK/antigen-specific function. One interpretation of this data is that cytotoxic cells are undergoing a transition from non-specific to specific cytolytic activity, and that clones with dual specificity represent an intermediate in this maturation pathway. The observation that expression of NK activity by CTL_x clones is reversible supports this view (Brooks, 1983c; Ronchese et al., 1985).

The regulatory factors that induce NK activity in cloned human and murine CTL are not well defined. In the murine system, stimulation of cloned CTL with specific antigen elicited NK activity by a process that was independent of IL 2, but appeared to require gamma IFN (Brooks et al., 1985). CTL can also acquire NK activity after exposure to crude supernatants from lectin-stimulated lymphocytes or certain tumor cell lines. Since these supernatants contain many lymphokines (including IL 2 and gamma IFN), attempts have been made to identify the specific molecule(s) involved in controlling expression of NK activity. Results from these studies indicate that a

factor distinct from IL 2 and gamma IFN (possibly a T cell differentiation factor) is required for the acquisition of new lytic specificities by CTL (Lopez-Botet et al., 1984; Ronchese et al., 1985; Brooks et al., 1985).

1.2 Purpose of the study

As noted above, PV causes a fatal infection in adult hamsters of the MHA strain, but not in those of the LSH or LVG strains. Buchmeier and Rawls (1977) observed that treatment of PV-infected LVG hamsters with cyclophosphamide (CY; a potent immunosuppressive drug) abrogated resistance to lethal PV infection. This result led to the hypothesis that cell-mediated immune responses are important in the control of PV replication, and that the lack of such a response in MHA strain hamsters could account for their susceptibility to PV infection. Subsequent studies revealed that MHA hamsters were unable to generate a delayed type hypersensitivity response after footpad challenge with PV. However, these normally susceptible animals survived infection by this route, and were found to be protected against later i.p. challenge with PV. These results suggested that MHA hamsters can mount a protective immune response to PV.

More recent studies have demonstrated that MHA hamsters have an additional spleen cell population for PV replication that is absent or present in reduced amounts in LSH hamsters. The available data suggest that this cell has NK activity, but further characterization has been difficult as the immunogenetic system of the hamster is poorly defined and there is a lack of antibodies to lymphocyte surface markers. In particular, there is a lack of information on the hemopoietic lineage to which these cells belong. This issue is of

importance as the target for PV replication could be an immature cell that has failed to differentiate because of a defect in lymphopoeisis in MHA (but not LSH) hamsters.

The goal of this study was to screen various strains of mice for differences in susceptibility to PV infection, and to determine their ability to mount cell mediated immune responses to the virus. During the course of these investigations it was observed that primary challenge of mice with PV resulted in the in vivo generation of NK and CTL responses. Furthermore, rechallenge of PV-primed mice with homologous virus elicited rapid memory NK and CTL activities. These primary and secondary cytotoxic effector cells were characterized in terms of their kinetics of development, cell surface phenotype, and susceptibility to inhibition by CY. In addition, experiments were undertaken to investigate the hypothesis that PV-induced NK activity is mediated by CTL precursors that become H-2 restricted and virus-specific later in infection.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Cell lines and cell culture

2.1.1 Suspension cell lines

Suspension cell lines were maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Gibco), 10 mM L-glutamine, 0.075% (w/v) $NaHCO_3$, 10 mM N-2-hydroxyethyl piperazine-N'-2-ethano- sulfonic acid (HEPES), 100 ug/ml streptomycin, and 100 U/ml penicillin.

YAC-1, a subline of the YAC lymphoma induced in A/Sn strain mice by Moloney leukemia virus (Kiessling et al.,1975), was used as a target cell for mouse NK cells. MLA-144, a gibbon ape lymphosarcoma, constituitively produces IL 2 and has been described in detail (Rabin et al., 1981; Brown et al., 1982).

2.1.2 Attached cell lines

BHK_{21} and Vero cell lines were maintained in plastic tissue culture flasks (Falcon, Oxnard, CA). Growth medium was alpha-minimal essential medium (MEM; Gibco) supplemented with 5% FCS, 0.075% $NaHCO_3$, and antibiotics. Murine fibroblast cell lines used as targets in ^{51}Cr release assays [including L929 ($H\text{-}2^k$), LTA ($H\text{-}2^k$), MC57G ($H\text{-}2^b$), PAK ($H\text{-}2^b$), and B10·D2 ($H\text{-}2^d$)] were also grown in MEM medium. L929 and LTA are sublines of parental L cells, a continuous line originally derived from connective tissue of a C3H/An mouse (Earle, 1943). PAK and B10·D2 cell lines (kind gift of Dr. P. Klein, University of Florida, Gainesville, FL) were derived from 20-methyl-cholanthrene induced tumors (Simrell and Klein, 1979; Klein, 1981).

2.2 Viruses and virus assays

PV (strain AN3739) is a member of the Arenavirus family and

was originally isolated from the blood of an Oryzomys albigularis rodent in the Pichinde valley of Colombia (Trapido and Sanmartin, 1971). Munchique virus (MV; Compans et al., 1981) is regarded as a strain of PV (Berge, 1975). Tacaribe virus (TV; kindly provided by Dr. R. Compans, University of Alabama, Birmingham, Alabama) is a member of the arenavirus family. It was first isolated from the fruit bat Artibeus literatus in Trinidad (Downs et al., 1963). Lymphocytic choriomeningitis virus (LCMV), strains WE and Armstrong, were a gift from Dr. J. McCormick, Centers for Disease Control, Atlanta, GA. Strain WE was isolated from a human case of non-bacterial meningitis (Rivers and Scott, 1936), while the Armstrong strain was isolated under unknown circumstances (Armstrong et al., 1934).

2.2.1 Propagation and titration of Arenaviruses

All arenaviruses (PV, LCMV, TV, and MV) were propagated in BHK_{21} cells as previously described (Mifune et al., 1971). Monolayers of BHK_{21} cells in 175 cm^2 tissue culture flasks (Nunclon, Denmark) were infected at a multiplicity of infection (MOI; number of pfu/cell) of 0.1- 0.2. Cells were refed and at two and three days after infection culture supernatants were collected on ice and centrifuged at 500 xg for 10 minutes. The virus-containing supernatant was aliquoted and frozen at -70°C until use.

PV, LCMV, TV, and MV were titered by the plaque assay method (Mifune et al., 1971). Serial 10-fold dilutions of virus were prepared on ice and 0.4 ml aliquots were applied to Vero cell monolayers in 60 mm tissue culture dishes (Falcon). Adsorption was for 60 minutes at 37°C in a humidified incubator. The virus inoculum was then aspirated and the monolayers were overlaid with 4 ml of

maintenance medium. Maintenance medium was 1.5% (w/v) agar (Difco, Detroit, MI) diluted 1:1 with Hanks balanced salt solution supplemented with 20% FCS, 2% (v/v) 100x BME amino acids (Gibco), 2% (v/v) 100x BME vitamins (Gibco), 4.0 mM L-glutamine, 200 ug/ml streptomycin, 200 U/ml penicillin, 4.5 g/L $NaHCO_3$, and 20 mM HEPES buffer. Plates were incubated at 37°C for 3 days and a second overlay of maintenance medium containing 1% (v/v) neutral red dye (Gibco) was then applied. Plates were returned to the 37°C incubator and plaques were counted 24 hours later.

2.2.2 Propagation and titration of vesicular stomatitis virus

Vesicular stomatitis virus (VSV), strain Indiana (Mudd-Summers), was grown in BHK_{21} cells (Rosenthal and Zinkernagel, 1980). Briefly, confluent monolayers of BHK_{21} cells in 175 cm^2 tissue culture flasks were infected with VSV at a MOI of 0.2- 0.3. Flasks were incubated at 37°C for 30- 45 minutes, refed with fresh medium, and returned to the incubator for 24 hours. Virus was harvested by freeze-thawing flasks once, collecting virus-containing supernatants, and centrifuging (500 xg for 10 minutes) to clear cellular debris. Virus stocks were aliquoted and frozen at -70°C until use. VSV stocks were routinely titered on Vero cells as described elsewhere (Holland and McLaren, 1959).

2.2.3 Tissue virus assay

Virus titers in the serum and organs of mice infected with PV or LCMV were determined. Organs were homogenized with sufficient phosphate buffered saline (PBS) to make a 10% (w/v) suspension. Homogenates were clarified by centrifugation (500 xg for 10 minutes), and the supernatants were titered by the plaque assay method (Mifune

et al., 1971). Samples were stored at -70°C prior to titration. Virus titers are expressed as the number of plaque forming units (pfu) per gram of organ or per milliliter of serum.

2.3 Generation of virulent PV strains

Virus stock was prepared by plaque purifying PV (strain AN3739) three times in Vero cells, followed by expansion in BHK_{21} cells as described above. Newborn B10.Br and CBA mice (less than 24 hours of age) were inoculated by the i.p. route with 2000 PFU of virus in a volume of 0.1 ml. Seven days later mice were sacrificed and pooled spleens were homogenized with sufficient PBS to make a 10% (w/v) suspension. The homogenized spleen pool was clarified by centrifugation (500 xg for 10 minutes) and used to infect additional suckling mice at a dose of 0.1 ml of spleen pool material per mouse. This process was continued through 8 passages.

Twenty B10.Br mice (4 weeks of age) were infected i.p. with 2000 PFU of original stock virus (designated prototype PV) or 8^{th} passage virus from the B10.Br strain and were observed daily for lethality. An additional 3 animals in each group were sacrificed at various times after infection and the virus content of pooled spleen homogenates was determined by plaque assay.

2.4 Delayed type hypersensitivity reaction

Inbred male Balb/c, CBA, C57BL/6, and B10.D2 mice (6- 8 weeks of age) were used in these studies. Mice were immunized in the left hind footpad with $6x10^6$ pfu of PV in a volume of 0.03 ml. Supernatant from uninfected BHK_{21} cells was inoculated into the right hind footpad as a control. Swelling in the left and right footpads was measured at daily intervals using dual gauge calipers. The percent increase in

footpad swelling was calculated using the formula of Tosolini and Mims (1971):

$$\frac{\text{Thickness of left hind footpad} - \text{Thickness of right hind footpad}}{\text{Mean thickness of hind footpads before inoculation}} \times 100$$

2.5 Generation of cytotoxic cell responses: immunization of adult mice with viruses

All mice used in this study were purchased from the Jackson Laboratories (Bar Harbour, ME) and were used between 6 and 9 weeks of age. Strains included C57BL/6 (H-2^b), C57BL/10 (H-2^b), B10.Br (H-2^k), CBA (H-2^k), Balb/c (H-2^d), and B10.D2 (H-2^d). Mice were housed in polycarbonate wire topped cages and fed Purina chow and water ad lib.

The mice received a primary immunization of virus by the intravenous (i.v.) route at the following doses: PV, $6x10^7$ pfu; LCMV (WE), $5x10^7$ pfu; LCMV (Armstrong), $1x10^4$ pfu; Munchique virus, $6x10^4$ pfu. Secondary immunization was carried out by reinfecting mice 5 to 7 weeks later with $6x10^7$ pfu of PV by the i.v. route.

2.6 Assays for cytotoxic cell activity

Virus infected mice were sacrificed by cervical dislocation and spleen cells were assayed for cytotoxic activity. CTL activity was determined against virus-infected, ^{51}Cr-labelled L929 (H-2^k), PAK (H-2^b), MC57G (H-2^b), and B10.D2 (H-2^d) target cells as described (Walker et al.,1984). Target cells were infected 24 to 48 hr before assay with PV or MV at an MOI of 10, or 24 hours before assay with LCMV or TV at a MOI of 0.2. Target cells were infected with vesicular stomatitis virus (VSV) 3 hours before assay at a MOI of 5. Target cells (usually $2x10^7$) were labelled with 300 microCuries (uCi) of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) in a volume of 1 ml for 90

minutes. After labelling cells were washed 5 times with medium and incubated with effector spleen cells in 96 well flat-bottomed microtiter plates (Nunclon, Denmark). Typical effector cell:target cell ratios were 40:1, 12:1, and 4:1. Assay duration was 7.5 hours for target cells infected with LCMV, TV, PV, or MV and 6 hours for VSV infected targets.

NK activity was determined by incubating spleen cells with the NK-sensitive YAC-1 cell line. Experimental conditions were identical to those described for the CTL assay except that the incubation time was 4 hours. The minimum (background) release of ^{51}Cr from target cells was determined by incubating the cells in medium without effector cells. Maximum ^{51}Cr release was determined by incubating target cells in 1N HCL. All test wells (including minimum release, maximum release, and sample wells with effectors and targets) were set up in duplicate, and the average number of cpm released from these test wells was used to calculate percent spontaneous and specific ^{51}Cr release. The number of cpm released from each of two individual test wells did not usually vary by more than five percent from the mean. The percent spontaneous ^{51}Cr release is defined as:

$$\frac{\text{cpm medium control}}{\text{cpm HCL control}} \times 100$$

The percent specific ^{51}Cr release is calculated as:

$$\frac{\text{cpm test sample} - \text{cpm medium control}}{\text{cpm HCL control} - \text{cpm medium control}} \times 100$$

Each experiment was repeated at least three times with concordant results

2.7 Treatment with CY

Cyclophosphamide (Procytox, Horner, Montreal) was

administered to mice by i.p. injection at a dose of 200 mg/kg of body weight. Eighteen hr later mice were given a primary immunization of $6x10^7$ pfu of PV by i.v. injection. In some experiments these mice were rechallenged with virus 5 weeks later. Secondary infection with PV was at a dose of $6x10^7$ pfu by i.v. injection. Mice were co-infected by i.v. injection of $6x10^7$ pfu of PV followed immediately by $9x10^4$ pfu of TV or LCMV, or $2x10^7$ pfu of VSV.

2.8 Secondary cytotoxic responses in vitro

A modification of the method described by Gardner and Blanden (1976) was used. Memory spleen cells were obtained from mice primed 5 weeks earlier with PV. These "responder" spleen cells were cultured with irradiated (1500R), PV infected, syngeneic peritoneal exudate "stimulator" cells. Peritoneal exudate cells were collected by lavage 2- 3 days after i.p. injection with 2 ml of sterile thioglycollate broth (Difco, Detroit, MI). Cells were mixed at a responder:stimulator ratio of 10:1. Usually, $1x10^7$ responders were incubated with $1x10^6$ stimulators at 37°C in an atmosphere of 10% CO_2 in 10 ml of RMPI medium supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UH), $5x10^{-5}$M 2-mercaptoethanol and antibiotics in upright 25 cm^2 flasks (Falcon). Cultures were harvested 5 days later and tested for CTL activity as described above.

2.9 Generation of interleukin 2-containing supernatants

2.9.1 Rat spleen cell conditioned medium

Supernatants of concanavalin A (Con A) stimulated rat spleen cells were prepared according to Gillis et al. (1978). Briefly, rat spleen cells were cultured at a density of $5x10^6$/ml in RPMI containing 5 ug/ml of Con A (Pharmacia, Uppsala, Sweden), 5% FCS, 10mM HEPES,

10mM L-glutamine, 0.075% $NaHCO_3$, 100 U/ml penicillin, and 100 ug/ml streptomycin. Forty-eight hours after initiation of culture, the supernatant was recovered following centrifugation at 500 xg for 20 minutes. Supernatants were passed through a 0.22 micron filter and stored at -20°C until use.

2.9.2 MLA-144 conditioned medium

Conditioned medium from the MLA-144 cell line was also used as a source of IL 2 activity. IL 2-containing supernatant was collected every 2- 3 days from actively growing cultures of MLA-144 cells. This conditioned medium was centrifuged at 500 xg for 10 minutes and stored at -20°C.

2.9.3 Highly purified murine IL 2

Highly purified murine IL 2 was a gift from Dr. V. Paetkau, University of Alberta, Edmonton, Alberta. A subclone (EL4.E1) of the murine T lymphoma EL4 described by Farrar et al. (1980) was used as a source of IL 2. The method used to purify IL 2 has been described in detail (Riendeau et al., 1983). IL 2 was shown to be pure by one and two dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Riendeau et al., 1983), and N-terminal amino acid sequencing (V. Paetkau, private communication). The activity of purified IL 2 was assayed on the IL 2 dependent MTL 2.8 CTL line as previously described (Gillis et al., 1978; Riendeau et al., 1983). The units of activity were calculated by the method of Farrar et al. (1980). One unit of IL 2 is defined as the amount which, in 1 ml, causes 30% of the maximum proliferative response of the MTL 2.8 cell line. The maximum proliferative response was determined in the same experiment by adding saturating levels of IL 2.

2.10 IL 2 assay

Purified IL 2 and IL 2-containing supernatants were assayed for their ability to induce proliferation of the H-Y cell line (provided by Dr. H. Hengartner, University of Zurich, Switzerland) by the method of Gillis et al. (1978). Proliferation of H-Y, a cloned NK cell line (Hengartner et al., 1982; Acha-Orbea et al., 1983; Bland et al., 1984; Rosenthal et al., 1984), has been shown to be IL 2 dependent using purified IL 1, IL 2, and IL 3 (Dr. K. Rosenthal, private communication). Serial two-fold dilutions of IL 2 samples were prepared and 100 ul of each dilution was delivered in duplicate to microwells. One hundred ul containing $1x10^4$ H-Y cells were added to the wells and cultures were incubated with 50 ul of ^{3}H-Tdr (1 uCi/well; New England Nuclear) for 6 hours. The cells were then harvested onto glass fibre strips with a MASH II harvester and processed for detection of radioactivity in a Beckman scintillation counter.

2.11 Limiting dilution assay of CTLp frequency

The method for determining CTLp frequency was similar to that of Askonas et al. (1982). Serial 1.5-fold dilutions of responder spleen cells were cultured with a constant number ($1x10^5$) of stimulator cells in 96-well flat-bottomed microtiter plates (Nunclon) in a final volume of 0.2 ml. Stimulator cells were irradiated (1500R) syngeneic peritoneal macrophages infected with PV at a MOI of 1 as described above. Culture medium (RPMI) was supplemented with 5% (v/v) lectin-free human T cell growth factor (Cellular Products Inc., Buffalo, N.Y.). Cultures were incubated for 5 days at 37°C in a humidified incubator and cytotoxicity was then determined against

^{51}Cr-labelled PAK ($H\text{-}2^b$) cells infected with PV. Target cells ($1X10^4$) in 0.05 ml of medium were added to each well. Plates were then incubated for 7.5 hours at 37°C. Spontaneous release of ^{51}Cr was determined by incubating target cells with stimulator cells alone. Wells were scored as positive if the cpm of ^{51}Cr release exceeded the spontaneous release by 2 standard deviations. Twenty-four wells were assayed at each cell dilution. CTLp frequency was determined by the maximum liklihood method of Porter and Berry (1964).

2.12 Neutralizing antibody

Fourteen days after secondary challenge with PV, mice were bled retrorbitally and serum was collected. Pooled sera were tested for neutralizing activity by the constant virus-varying serum technique (Peters and Bellanti, 1980). In brief, 400 pfu of PV in a volume of 0.2 ml were incubated with serial 2-fold dilutions of sera at 37°C for 60 minutes. Each dilution (0.1 ml) was then plated on Vero cell monolayers and assayed for infectious virus as described (Mifune et al., 1971).

2.13 Antibodies

2.13.1 Treatment of spleen cells with antibody and complement

PV immune-spleen cells were incubated with monoclonal anti-Thy-1.2, anti-Lyt-1.2, or anti-Lyt-2.2 antibodies (New England Nuclear). Anti-Thy-1.2 was diluted 1/500 in PBS, while anti-Lyt-1.2 and anti-Lyt-2.2 were diluted 1/100. Anti-asialo GM1 (anti-asGM1) antiserum (Wako Pure Chemicals, Dallas, TX) was used at a 1/25 dilution (except where indicated). Spleen cells ($7x10^6$) were incubated with 0.05 ml of antibodies, washed once, and resuspended in 1 ml of a 1/10 dilution of Low-Tox rabbit complement (Cedarlane

laboratories, Hornby, Ontario). Complement treatment was for 30 minutes at 37°C. Cells were washed 3 times with medium and tested for cytotoxicity in a ^{51}Cr release assay as described above.

2.13.2 Use of anti-asGM1 antiserum *in vivo*

To abrogate cytotoxic cell responses *in vivo*, C57Bl/6 mice were injected i.v. with 0.2 ml of a 1/10 dilution of anti-asGM1 antiserum 6 hours before primary or secondary infection with PV. In some experiments treatment with antiserum continued at daily intervals until mice were sacrificed and spleen cells were assayed for cytotoxic activity.

2.13.3 Production of glycolipid micelles.

Purified human glycolipids monoganglioside GM 1 (GM 1) and gangliotetraosylceramide (asialo GM 1) were purchased from Supelco Corp. (Oakville, Canada). Egg lecithin (type VIII) and cholesterol were purchased from Sigma (St. Louis, MO). A mixture of glycolipid (either GM 1 or asGM 1):lecithin:cholesterol (1:4:10 w/w/w) was prepared in a chloroform:methanol buffer (2:1 v/v) according to the method of Graf and Rapport (1965). The solvent was evaporated under a stream of nitrogen gas at 37°C and the residue was resuspended in absolute ethanol for storage at -4°C.

The ability of anti-asGM 1 antibodies to recognize asGM 1 antigen was determined using a microflocculation assay (Kasai *et al.*, 1980). Briefly, the glycolipid:lecithin:cholesterol mixture was dispersed in PBS by sonication for 3 minutes on ice, and 50 microliter aliquots containing 7 ug of glycolipid, 28 ug of lecithin, and 70 ug of cholesterol were added to round bottom microtiter wells which contained an equal volume of anti-asGM 1 antiserum. Plates were

incubated for 2 hrs at room temperature and the highest twofold dilution of antiserum causing a visible agglutination reaction was determined.

Anti-asGM 1 antibodies were absorbed with antigen-containing micelles according to the method of Naiki et al. (1974). Graded amounts of the micelles were mixed with anti-asGM 1 antiserum (final dilution 1:25) in a total volume of 1 ml of PBS. Antigen-antibody mixtures were incubated overnight at 4°C and then centrifuged at 12,000 xg for 45 minutes to remove immune complexes. The upper 0.9 ml of supernatant was carefully removed and stored at -4°C.

CHAPTER THREE

RESULTS

3.1 Susceptibility of inbred mice to infection with PV and generation of virulent PV strains

The splenic target cells supporting PV replication in susceptible (MHA) hamsters co-purify with cells having NK activity (Gee et al., 1981b). Because characterization of this putative cell population has been hampered by a lack of antibodies to lymphocyte surface markers in the hamster, attempts were made to develop a similar model using inbred strains of mice.

Adult inbred mice of various haplotypes were screened for differences in susceptability to PV infection. Groups of 5 Balb/c ($H\text{-}2^d$), CBA ($H\text{-}2^k$), and C57BL/10 ($H\text{-}2^b$) mice (6- 8 weeks of age) were infected with PV and observed for lethality. Inoculation was by the i.p. or i.c. route at a dose of $1x10^7$ pfu of PV per mouse, or i.v. at a dose of $4x10^7$ pfu of PV per mouse. All animals in each test group survived infection and appeared healthy until the experiment was terminated 4 months later.

Because prototype PV was not lethal for adult mice, attempts were made to generate a more virulent PV strain by forced passage through suckling mice. Plaque purified PV (2000 pfu) was inoculated i.p. into newborn B10.Br mice and seven days later pooled spleen homogenates were prepared and inoculated into a second newborn litter. This process was continued through 8 consecutive passages. Two groups of 10 B10.Br mice (4 weeks of age) were inoculated with 2000 pfu of either prototype PV (strain AN3739) or the passaged virus from B10.Br mice and observed for lethality. Both viruses were avirulent and all mice were alive 2 months after infection when the experiment was

terminated.

The ability of prototype PV and the passaged virus to replicate in the spleens of 4 week old B10.Br mice was compared (table 1). Passaged and prototype PV viruses could not be detected in spleen homogenates longer than 3 and 6 days after infection, respectively. Furthermore, both viruses appeared to replicate equally well, as the amount of virus recovered from the spleens of adult B10.Br mice infected with passaged or prototype strains was similar.

3.2 Cell-mediated immune responses to PV in various strains of mice

The outcome of PV infection appeared to be similar in all strains of inbred mice tested. This prompted a search for strain-related differences in cell-mediated immune responses to the virus.

3.2.1 The delayed-type hypersensitivity reaction

Various strains of adult inbred mice (Balb/c, C57BL/10, B10.D2, and CBA) were immunized in the hind footpad with PV and swelling was measured daily with dual gauge calipers. The data shown in figure 1 are for the C57B1/10 and CBA strains. Swelling peaked 7 days after infection, and the magnitude of the response was similar in all strains tested.

3.2.2 Primary cytotoxic cell responses

C57BL/6 mice were injected i.v. at daily intervals with PV and their spleen cells were assayed for cytotoxic activity against YAC-1 cells and against virus-infected and uninfected target cells. After infection, a marked augmentation in cytolytic activity against NK-sensitive YAC-1 cells was observed immediately. This virus-induced NK activity peaked 3- 4 days after infection and declined thereafter (figure 2A).

TABLE 1

REPLICATION OF PROTOTYPE AND ADAPTED PV STRAINS IN THE SPLEENS OF ADULT INBRED B10.Br MICE

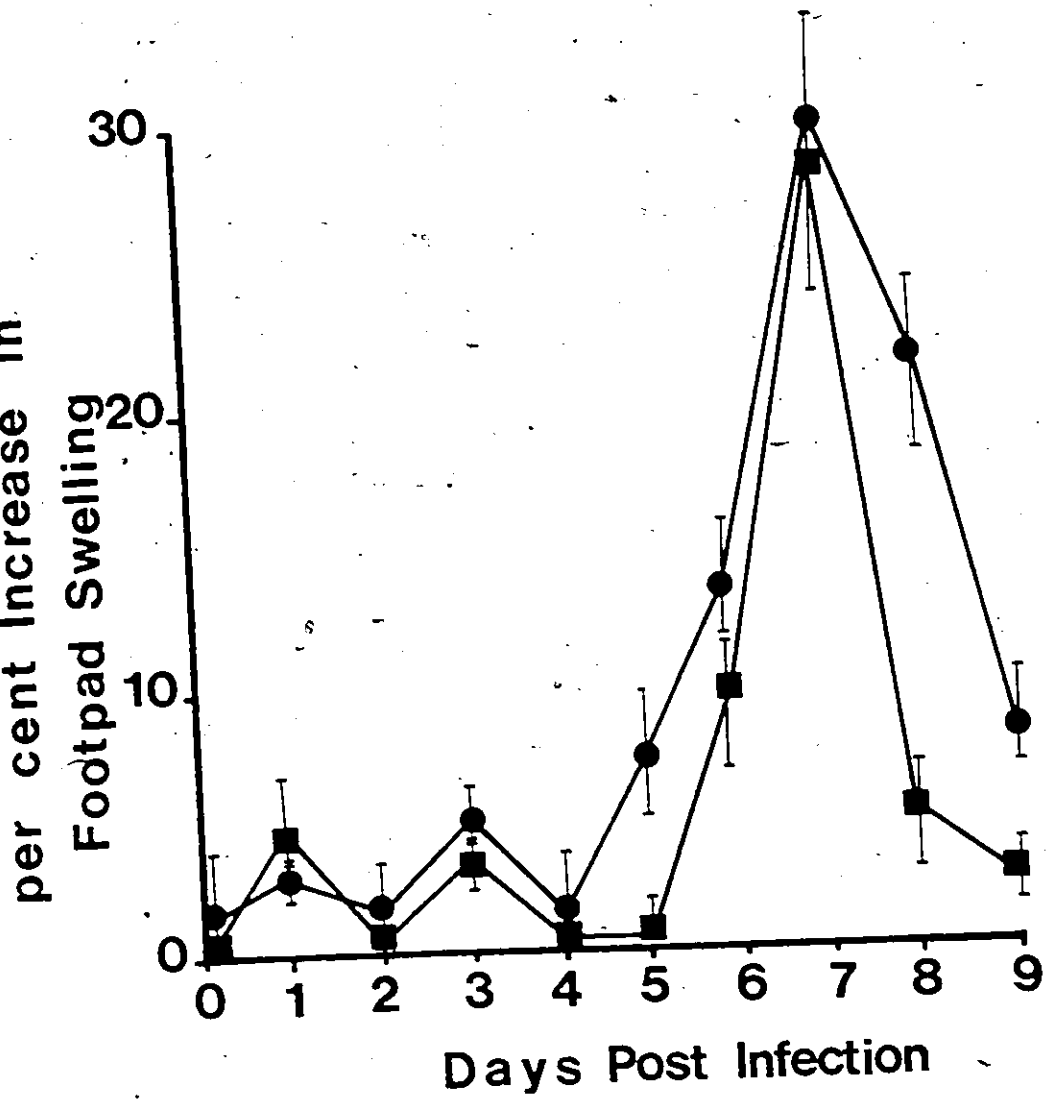
Virus Strain Inoculated[a]	Day After Inoculation				
	1	3	6	9	12
ADAPTED	2.5[b]	2.5	<2[c]	<2	<2
PROTOTYPE	2.5	2.5	2.2	<2	<2

[a] 2000 pfu of prototype (strain AN3739) or B10.Br adapted PV were inoculated into 4 week old male B10.Br mice.

[b] Log_{10} pfu of adapted or prototype PV per gram of pooled spleen homogenate from each of three mice.

[c] <2 pfu of PV per gram of spleen, the lower limit of detection in this assay.

Figure 1. DTH responses to PV in mice. Groups of 5 C57Bl/10 (■—■) and CBA (●—●) mice were inoculated in the left hind footpad with $6x10^6$ pfu of PV in a volume of 0.03 ml. Medium from uninfected BHK cells was injected into the right hind footpad as a control. Swelling was measured at various intervals afterwards with dual gauge calipers and the percent increase in footpad swelling was calculated as described in Materials and Methods. Each point represents the average percent increase in footpad swelling from a group of five mice +/- the standard error mean.


per cent Increase In Footpad Swelling
30
20
10
0
0 1 2 3 4 5 6 7 8 9
Days Post Infection

Pichinde virus also elicited a potent cytolytic response against virus-infected syngeneic cells. This activity did not appear until about 5 days after infection and peak activity was observed on days 6 or 7 (figure 2A); uninfected syngeneic target cells were not lysed. Target cells infected with PV 24 or 48 hours before assay were most sensitive to lysis by these cytotoxic cells (figure 3). Effector spleen cells obtained on day 7 after infection were characterized with respect to H-2 restriction and virus specificity. The data shown in table 2 indicate that the cytolytic activity was H-2 restricted; effector cells from CBA mice lysed syngeneic virus-infected L929 ($H\text{-}2^k$) cells more efficiently than histoincompatible MC57G ($H\text{-}2^b$) or B10.D2 ($H\text{-}2^d$) target cells that had been infected with PV. Similarily, effector cells from C57BL/6 ($H\text{-}2^b$) and Balb/c ($H\text{-}2^d$) immune mice lysed virus-infected MC57G and B10.D2 target cells, respectively, more efficiently than histoincompatible target cells (table 2). Furthermore, these data demonstrate that a variety of inbred mouse strains are responsive and capable of generating an H-2 restricted cytolytic response to PV.

Effector spleen cells obtained 7 days after infection with PV only lysed PV-infected syngeneic targets and did not lyse targets infected with LCMV(ARM), LCMV(WE), or TV (table 3). Thus, effector cells obtained 7 days after PV infection were H-2 restricted, virus-specific CTL. Spleen cells from mice infected with PV also lysed target cells infected with MV, which is a strain of PV.

3.2.3 Memory cytotoxic cell responses

Sera were obtained from mice 14 days after secondary challenge with PV and were assayed for their capacity to neutralize

Figure 2. Kinetics of CTL and NK responses to PV. A. Primary responses: pairs of C57Bl/6 mice were infected with PV at daily intervals and their spleen cells were assayed for cytolytic activity against YAC-1 (○) and PV-infected MC57G (●) target cells as described in Materials and Methods. The values at day 0 represent cytolytic activity from the spleens of uninfected mice. B. Secondary responses: C57Bl/6 mice were rechallenged at daily intervals with homologous PV and the cytolytic activity of spleen cells was determined against YAC-1 (○) and PV-infected MC57G (●) target cells. The lymphocyte:target cell ratio in both A and B is 40:1. Spontaneous ^{51}Cr release from target cells was less than 20%. Cytolytic activity against uninfected MC57G target cells was less than 10%.

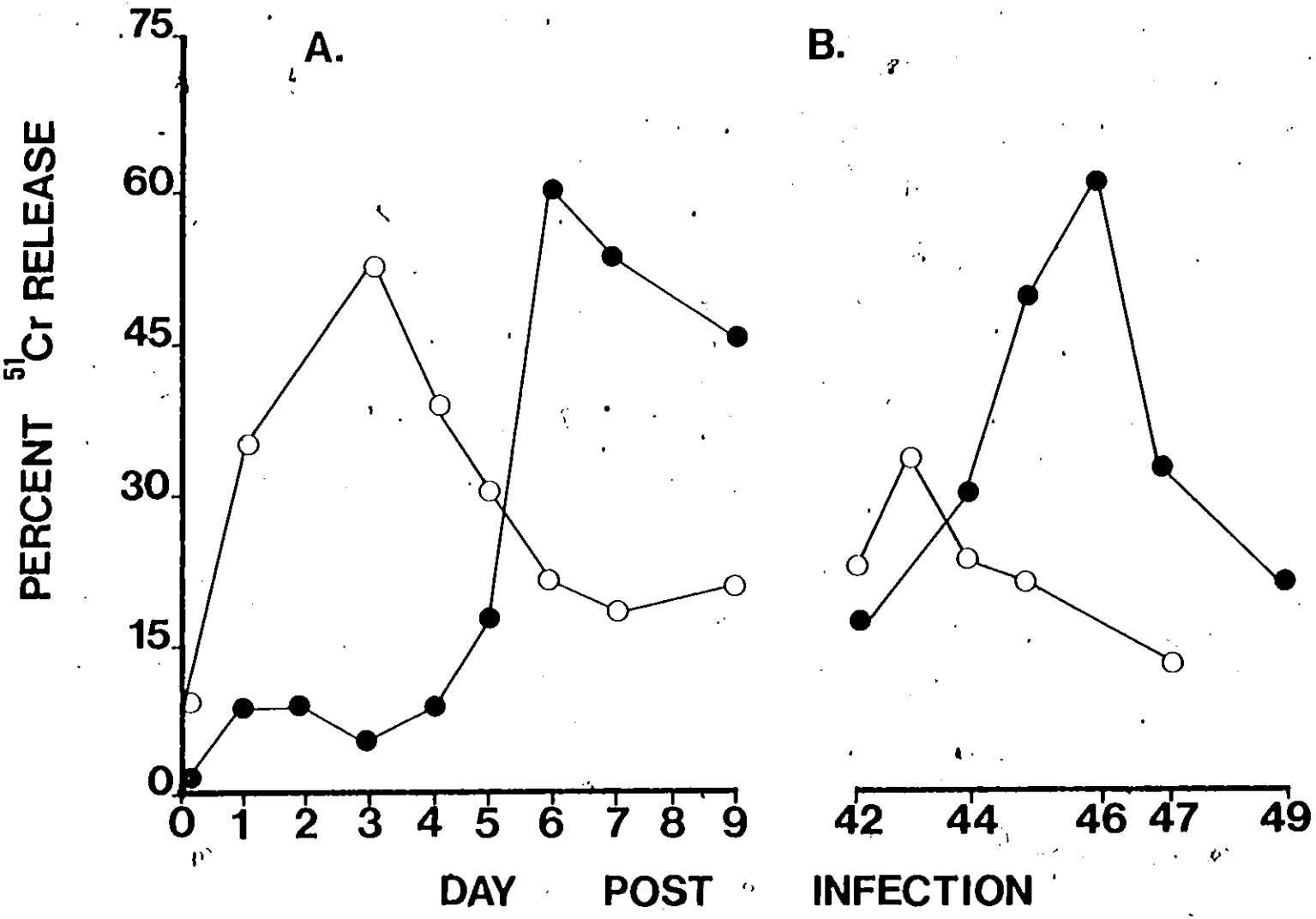

A.
B.
PERCENT ^{51}Cr RELEASE
75
60
45
30
15
0
0 1 2 3 4 5 6 7 8 9
DAY POST
42 44 46 47 49
INFECTION

Figure 3. Infection of MC57 target cells with PV: Effect of time of infection on susceptibility to CTL lysis. MC57 target cells were infected with PV at various times before assay for susceptibility to CTL lysis. Effector spleen cells were taken from C57Bl/6 mice 7 days after primary infection with PV. Assay duration was 7.5 hrs and effector:target cell ratio was 40:1. Spontaneous release of ^{51}Cr from all target cells was less than 20%.

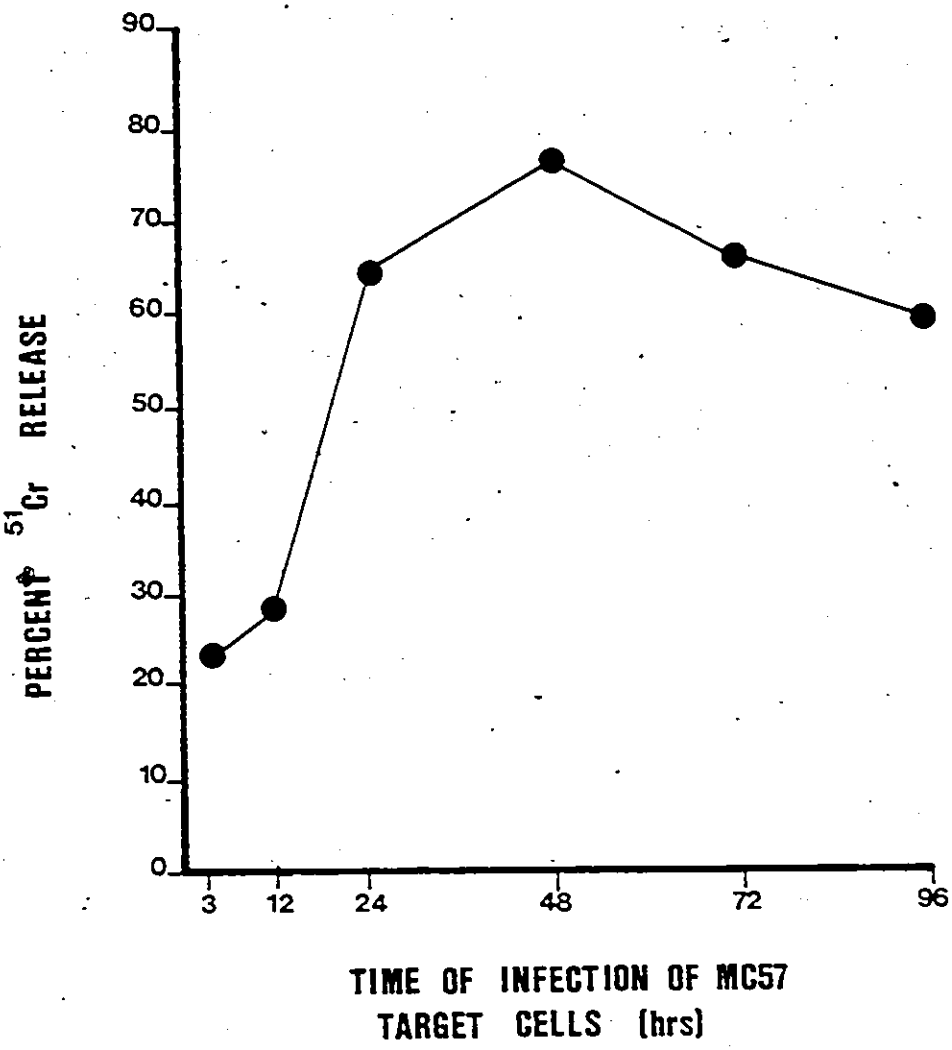

90
80
70
60
50
40
30
20
10
0
PERCENT ^{51}Cr RELEASE
3
12
24
48
72
96
TIME OF INFECTION OF MC57
TARGET CELLS (hrs)

TABLE 2

H-2 RESTRICTION OF PRIMARY AND SECONDARY CTL RESPONSES TO PV[a]

Effector[b]	Haplotype	Percent Specific ^{51}Cr Release From PV Infected		
		L-929 ($H\text{-}2^k$)	B10.D2 ($H\text{-}2^d$)	MC57G ($H\text{-}2^b$)
Primary CBA	($H\text{-}2^k$)	74	20	10
Primary C57Bl/6	($H\text{-}2^b$)	13	22	68
Primary Balb/c	($H\text{-}2^d$)	3	67	9
Secondary CBA	($H\text{-}2^k$)	58	2	2
Secondary C57Bl/6	($H\text{-}2^b$)	5	15	48
Secondary Balb/c	($H\text{-}2^d$)	<1	54	5

[a]Primary and secondary PV immune spleen cells were obtained 7 and 4 days post infection, respectively.

[b]Lymphocyte:target cell ratio 40:1

TABLE 3

VIRUS-SPECIFICITY OF ANTI-ARENAVIRUS CTL

CBA/J Mice Infected With:	Effector: Target Cell Ratio	Percent Specific ^{51}Cr Release From L929 Cells:[b]				
		Uninfected	PV	LCMV(WE)	LCMV(ARM)	TV
PV	40:1	2	60	12	9	6
	12:1	3	53	4	8	9
	4:1	3	20	<1	5	4
MV	40:1	<1	42	24	11	12
	12:1	<1	31	17	5	10
	4:1	<1	17	7	5	2
LCMV(WE)	40:1	<1	7	49	43	3
	12:1	<1	6	44	41	2
	4:1	<1	<1	26	20	2
TV	40:1	<1	5	11	3	40
	12:1	<1	<1	13	3	28
	4:1	1	2	4	5	12

[a] CBA/J mice were infected via the i.v. route with PV (6×10^7 pfu), MV (6×10^4 pfu), LCMV (WE) (5×10^7 pfu), or TV (9×10^4 pfu). Seven days later spleen cells were removed and cytotoxicity was tested against the syngeneic virus-infected target cells indicated.

[b] Assay time was 7.5 hr and lymphocyte:target cell ratio was 40:1. Spontaneous release was less than 25% for all target cells.

the virus. Neutralizing antibodies were not found (data not shown), which substantiates earlier findings by others (Trapido and Sanmartin, 1971; Chanas et al., 1980).

It was reasoned that because the mice did not make detectable neutralizing antibodies to PV, and because virus neutralization may be a factor in preventing a secondary CTL response in vivo, the mice might display a CTL response when rechallenged with homologous virus. Thus, C57BL/6 mice were reinjected with PV 5- 7 weeks after primary infection, and the spleen cells from these animals were assayed for cytolytic activity. The results are shown in figure 2B. In contrast to primary infection, rechallenge of the mice with PV resulted in a slight but significant increase in NK activity against YAC-1 target cells 1 day after infection; this activity rapidly returned to prechallenge levels.

Cytolytic activity against syngeneic virus-infected target cells was detected 2 days after rechallenge with PV (figure 2B). This activity peaked on day 4 and the magnitude of the response was comparable with that observed for CTL responses after primary infection with PV. To further characterize the cell population mediating the killing, spleens were removed from C57BL/6, CBA, and Balb/c mice 4 days after secondary rechallenge with PV and assayed for their ability to lyse H-2 compatible and incompatible virus-infected target cells. As shown in table 2, significant levels of lysis were observed only against syngeneic virus-infected target cells.

These results demonstrate that secondary infection of mice with homologous PV results in a more rapid generation of cellular immune responses. Furthermore, the temporal relationship between

virus-induced NK and CTL responses was maintained after both primary and secondary infection with PV, which suggested that a precursor-product relationship might exist between these two cell populations.

3.3 Cell surface antigens expressed on NK cells and CTL

The lineage relationship between PV-induced NK cells and CTL was investigated by comparing their cell surface phenotypes.

3.3.1 Cell surface phenotype of CTL: Thy-1, Lyt-1, and Lyt-2

Effector spleen cells were obtained 7 days after primary PV infection or 4 days after secondary infection and were tested for surface expression of the Thy-1.2, Lyt-1.2, and Lyt-2.2 antigens. As shown in table 4, treatment of primary and secondary effector cells with anti-Thy-1.2 or anti-Lyt-2.2 antibodies and complement abrogated cytotoxic activity against PV-infected syngeneic target cells. The activity was also partially reduced by treatment with anti-Lyt-1.2 and complement.

3.3.2 Expression of the Thy-1 surface antigen on virus-induced NK cells

Endogenous NK cells have been shown to express low or no Thy-1 surface antigen (Herberman et al., 1977; Pollack et al., 1979; Koo et al., 1980). It was reasoned that if NK cells differentiate to become virus-specific CTL, then increasing numbers of PV-induced NK cells should express Thy-1.2 as the infection progresses. Accordingly, C57BL/6 mice were injected i.v. at daily intervals with PV and spleen cells mediating lysis of YAC-1 target cells were assayed for sensitivity to treatment with anti-Thy-1.2 and complement. As shown in table 5, only a small fraction of virus-induced NK cells (5-15%) were eliminated with monoclonal anti-Thy-1.2 and complement when

tested two days after primary infection. By 4 days post-infection, however, the majority of virus-augmented NK activity was mediated by Thy-1.2 positive cells (Table 5).

As with the primary NK response, the secondary NK response rapidly acquired sensitivity to anti-Thy-1.2 and complement. Thus, by 2 days after PV rechallenge, the majority of virus-induced NK activity was abrogated by this treatment (Table 5).

3.3.3 Expression of the asialo-GM1 surface antigen on CTL precursors

Expression of the NK cell marker asialo-GM1 (asGM1) on CTL precursors (CTLp) was determined. Memory spleen cells were treated with anti-asGM1 antibodies and complement before restimulation in vitro with virus-infected stimulator cells. As shown in Table 6, this treatment prevented generation of a secondary in vitro CTL response.

To investigate the possibility that T helper cells rather than CTLp were eliminated with anti-asGM1 and complement, helper factors were added back to treated responder spleen cell cultures. As shown in table 7, neither IL 2-containing rat Con A supernatant nor irradiated memory spleen cells from PV-primed mice could restore CTL activity. This result suggests that anti-viral CTLp are sensitive to anti-asGM1 and complement-mediated lysis.

3.3.4 Effect of in vivo treatment with anti-asGM1 antiserum on generation of primary and secondary NK and CTL responses

To determine whether anti-asGM1 antiserum could eliminate cytotoxic responses in vivo, C57BL/6 mice were injected i.v. with antibodies and infected 6 hours later with PV. This treatment eliminated the primary PV-induced NK response but not CTL activity (table 8). Similarly, a single injection of antibodies before

TABLE 4

SURFACE PHENOTYPE OF PRIMARY AND SECONDARY ANTI-PV CTL[a]

Antibody	Complement	Primary CTL Response[b]			Secondary CTL Response		
		40:1[c]	12:1	4:1	40:1	12:1	4:1
-	-	88	60	41	34	25	13
-	+	79	56	40	ND[d]	-	-
Anti-Thy-1.2	+	7	8	6	6	4	2
Anti-Lyt-1.2	+	36	15	7	14	11	8
Anti-Lyt-2.2	+	12	8	9	3	5	3

[a]Primary and secondary C57Bl/6 PV immune spleen cells were obtained 7 and 4 days post infection, respectively.

[b]Data represent percent specific ^{51}Cr release from PV-infected MC57G target cells; assay time was 7.5 hr, spontaneous release was <25%.

[c]Lymphocyte:target cell ratio.

[d]ND = not done.

TABLE 5
INCREASED EXPRESSION OF THY-1 ANTIGEN ON VIRUS-INDUCED NK CELLS AFTER PRIMARY AND SECONDARY PV INFECTION

Response	Days Post Infection	Experiment 1			Experiment 2		
		untreated	treated[b]	(%reduction)[c]	untreated	treated	(%reduction)
Primary	2	21	20	(5)	14	12	(15)
Primary	4	26	11	(57)	10	<1	(100)
Secondary	1	28	33	(0)	14	6	(57)
Secondary	2	25	7	(72)	12	<1	(100)

[a]PV was administered to naive or PV-immune C57Bl/6 mice at various intervals before sacrifice. Treated or untreated effector spleen cells were then tested for cytotoxicity against YAC-1 target cells. Values shown represent the percent specific ^{51}Cr release at lymphocyte:target cell ratio of 40:1 in a 4 hr assay. Data shown are adjusted for virus-induced NK activity by subtracting the endogenous cytotoxicity in respective control treated and untreated mice. Complement alone did not reduce cytotoxicity; spontaneous release was less than 15 percent.

[b]Spleen cells were treated with monoclonal anti-Thy-1.2 and complement as described in Materials and Methods.

[c]Percent reduction in cytolysis was calculated using the formula described in the legend to table 11.

TABLE 6

PRETREAIMENT OF RESPONDER SPLEEN CELLS WITH ANTI-asGM1 ANTIBODIES AND COMPLEMENT PREVENTS THE IN VITRO GENERATION OF CTL

Responder Cells Treated With:[a]		Stimulator Cells Infected With PV	Percent Specific ^{51}Cr Release:[c]	
Anti-asGM1[b]	C'		PAK-uninfected	PAK-PV
-	-	-	13	34
-	-	+	27	95
-	+	+	23	82
+	+	+	14	25

[a] C57Bl/6 mice were infected with PV ($6x10^7$ pfu i.v.). Five weeks later spleen cells were removed and incubated in vitro with irradiated (1500R) uninfected or infected macrophages. Cultures were tested for cytotoxic activity 5 days later. Effector cell concentrations were not readjusted prior to assay.

[b] In some cases, responder spleen cells ($1x10^7$) were pretreated with anti-asGM1 antibodies (diluted 1:25) and complement.

[c] Assay duration was 7.5 hr; spontaneous release was less than 25 %.

TABLE 7

IL 2-CONTAINING SUPERNATANTS OR PV-PRIMED HELPER SPLEEN CELLS DO NOT RESTORE CYTOTOXIC RESPONSES TO ANTI-asGM1 PLUS C' TREATED RESPONDER CELLS

Responder Cells Treated With:[a]		Stimulator Cells Infected With PV	Source of Helper Activity[c]	Percent Specific ^{51}Cr Release From:[d]	
Anti-asGM1[b]	C'			PAK-uninfected	PAK-PV
-	-	-	-	15	27
-	-	+	-	15	61
-	+	+	-	9	73
+	+	+	-	<1	3
+	+	+	Con A sup.	<1	3
+	+	+	Irradiated Helper Cells	1	7

[a] C57Bl/6 mice were infected with PV ($6x10^7$ pfu i.v.). Five weeks later spleen cells were removed and incubated in vitro with irradiated (1500R) stimulator cells that were either uninfected or infected with PV. Cultures were tested for cytotoxicity 5 days later. Effector cell concentrations were not adjusted prior to assay.

[b] In some cases, responder spleen cells ($1x10^7$) were pretreated with anti-asGM1 antibodies and complement.

[c] Helper activity was supplied to antibody and complement treated cultures by adding 10% (v/v) rat Con A sup. or $1x10^6$ irradiated (2000R), PV-primed, memory spleen cells from C57Bl/6 mice.

[d] Assay duration was 7.5 hr; spontaneous ^{51}Cr release was less than 20%.

Table 8

TREATMENT WITH ANTI-asGM1 ANTIBODIES PRIOR TO PV INFECTION ABROGATES THE NK CELL RESPONSE BUT DOES NOT INHIBIT CTL GENERATION

Response[a]	Day Post Infection	Anti-asGM1[b] Antibodies	Percent Specific ^{51}Cr Release:[c] YAC-1	PAK-PV	PAK-uninfected
PRIMARY	4	−	64	19	ND[d]
		+	2	8	ND
PRIMARY	7	−	24	76	ND
		+	8	67	ND
SECONDARY	1	−	43	70	<1
		+	<1	4	<1
SECONDARY	4	−	26	92	5
		+	8	83	3

[a]Naive or PV-immune C57Bl/6 mice were infected with PV ($6x10^7$ pfu i.v.) at various intervals prior to sacrifice and spleen cells were then assayed for cytotoxic activity.

[b]Anti-asGM 1 antibodies (diluted 1:10) were injected i.v. in a volume of 0.2 ml 6 hr before primary or secondary infection with PV.

[c]Effector:target cell ratio was 40:1 and assay duration was 4 hr for ^{51}Cr YAC-1 targets and 7.5 hr for PAK targets; spontaneous release of ^{51}Cr from all target cells was less than 25 percent.

[d]ND = not done.

secondary infection with PV abrogated memory NK activity without affecting CTL generation.

It seemed possible that the CTL response was spared after a single injection of anti-asGM1 because the amount of antibody administered was insufficient or the timing of the injections was inappropriate. Accordingly, anti-asGM1 antibodies were administered before primary or secondary infection with PV, and then at daily intervals until spleen cells were assayed for CTL activity. As shown in table 9, this regimen abrogated the primary and secondary CTL responses. Thus, in vivo injection of anti-asGM1 prevented the generation of NK and CTL responses to PV.

3.3.5 Titration of anti-asGM1 on virus-induced NK cells and CTL

The relative amount of asGM1 antigen on virus-induced NK cells and CTL was compared by titrating the antiserum on both cell populations. Spleen cells were removed at 4 and 7 days after primary infection with PV as a source on NK and CTL effectors, respectively. These cells were incubated with serial 4-fold dilutions of antibodies, treated with complement, and assayed for cytotoxicity on the appropriate target cell. As shown in table 10, the dilution resulting in a 50% reduction in cytotoxicity was similar for NK cells and CTL (approximately 1/400). One interpretation of this result was that virus-induced NK and CTL effectors expressed an equivalent amount of asGM 1 antigen. Alternatively, it was possible that the antiserum was not monospecific for asGM 1, but also contained contaminating antibodies to pan-T cell markers. To test this possibility, studies were undertaken to show that the antiserum recognized asGM 1 antigen, and that absorption of the antibodies with purified antigen removed

TABLE 9

ABROGATION OF PRIMARY AND SECONDARY CTL RESPONSES TO PV BY DAILY ADMINISTRATION OF ANTI-asGM1 ANTIBODIES

Response[a]	Anti-asGM1 Antibody[b]	Lymphocyte: Target Cell Ratio	Percent Specific ^{51}Cr Release[c] PAK-uninfected	PAK-PV
PRIMARY	-	40:1	16	81
		12:1	11	82
		4:1	8	66
PRIMARY	+	40:1	3	<1
		12:1	4	2
		4:1	8	3
SECONDARY	-	40:1	5	82
		12:1	5	74
		4:1	9	36
SECONDARY	+	40:1	<1	1
		12:1	4	3
		4:1	4	2

[a] Naive or PV-immune C57Bl/6 mice were infected with $6x10^7$ pfu of PV i.v. Seven days after primary infection or 4 days after secondary infection mice were sacrificed and spleen cells were tested for cytotoxicity.

[b] Anti-asGM1 antibodies (1:10 dilution) were administered i.v. in a volume of 0.2 ml 6 hr before infection with PV, and then at daily intervals until assay.

[c] Assay time was 7.5 hr against syngeneic PAK target cells. Spontaneous release of ^{51}Cr was less than 25 percent.

TABLE 10
TITRATION OF ANTI-asGM1 ANTISERUM ON PV-INDUCED NK AND CTL EFFECTORS[a]

Reciprocal Dilution of Anti-asGM1[b]	complement	Cytotoxic Cell Response After PV Infection					
		Primary NK	(%reduction)	Primary CTL	(%reduction)	Secondary CTL	(%reduction)
–	–	41[c]	(0)[d]	68	(0)	87	(0)
25	–	30	(25)	ND[e]	(—)	ND	(—)
25	+	<1	(100)	2	(97)	4	(99)
100	+	2	(95)	4	(94)	43	(51)
400	+	21	(49)	44	(35)	75	(14)
1600	+	62	(0)	78	(0)	82	(6)

[a] C57Bl/6 mice were infected with PV (7×10^6 pfu i.v.), and spleen cells removed 4 days later were used as primary NK effectors; cytotoxicity was determined against ^{51}Cr-labelled YAC-1 cells. Spleen cells removed 7 days after primary infection or 4 days after secondary infection were used as primary and secondary CTL, respectively. CTL activity was determined against ^{51}Cr-labelled PAK-PV target cells.

[b] Spleen cells were treated with serial 4-fold dilutions of anti-asGM1 and complement. Treatment of spleen cells with complement alone did not reduce cytotoxicity.

[c] Values represent percent specific ^{51}Cr release at an effector:target cell ratio of 40:1. Spontaneous release of ^{51}Cr from target cells did not exceed 20 percent. PV-specific CTL did not cause significant ^{51}Cr release from uninfected PAK cells.

[d] Percent reduction in cytolysis was calculated using the formula set out in the legend to figure 11.

[e] ND = not done.

reactivity against PV-induced NK cells and CTL.

Reactivity of the antiserum with purified asGM 1 was demonstrated using a microflocculation assay as described in the Materials and Methods section. The highest dilution of anti-asGM 1 antiserum causing visible agglutination of micelles containing purified human asGM 1 was 1/32. Micelles containing purified human monoganglioside (GM 1) were not agglutinated by this antiserum.

As shown in figure 4, the ability of anti-asGM 1 antibodies and complement to inhibit the cytolytic activity of PV-induced NK and CTL effectors could be reduced in a dose-dependent fashion by absorbing the antibodies with graded amounts of purified asGM 1. Micelles containing the auxillary lipids lecithin and cholesterol (but not asGM 1) did not absorb out antibody reactivity against PV-induced NK cells. Taken together, these results suggest that the antiserum was specific for asGM 1. Furthermore, approximately equal amounts of the antigen appear to be expressed on the surface of virus-induced NK cells and CTL.

These findings differ from those of other investigators, who showed allospecific CTL to be considerably more resistant to treatment with anti-asGM1 and complement than endogenous NK cells (Kasai et al., 1980a; Young et al., 1980). Thus, it seemed possible that allospecific CTL would be less sensitive to anti-asGM1 and complement treatment when compared to virus-specific CTL. Accordingly, spleen cells were removed from C57Bl/6 (H-2^b) mice that had been immunized with PV or allogeneic P815 (H-2^d) tumor cells, treated with 4-fold dilutions of anti-asGM1 plus complement, and assayed for cytotoxic activity against the appropriate target cell (table 11). The

Figure 4. Absorption of anti-asGM 1 antibodies with asGM 1 antigen. Anti-asGM 1 antibodies (diluted 1:25) were absorbed with graded amounts of micelles containing asGM 1:lecithin: cholesterol (1:4:10 w/w/w) as described in the Materials and Methods section. Spleen cells taken from C57Bl/6 mice at 4 and 7 days after primary PV infection were used as a source of NK and CTL effectors, respectively; cytotoxic cells ($1x10^7$) were incubated with anti-asGM 1 antibodies or normal rabbit serum (NRS) and complement. The remaining NK cells were tested for cytotoxicity against YAC-1 target cells (●), while residual CTL activity was determined against syngeneic, PV-infected PAK target cells (■). Rabbit anti-asGM 1 antibodies were also absorbed with micelles containing lecithin and cholesterol but no asGM 1. NK cells treated with these antibodies and complement were then assayed for cytotoxic activity against YAC-1 targets (▲). Percent inhibition of target cell lysis by NK and CTL effectors was calculated using the formula:

$$100-\left[\frac{\%\ \text{specific}\ ^{51}\text{Cr release (anti-asGM 1 plus C')}}{\%\ \text{specific}\ ^{51}\text{Cr release (NRS plus C')}}\ x100\right]$$

Assay duration was 4 hrs for YAC-1 target cells and 7.5 hrs for PAK-PV target cells. Spontaneous release of ^{51}Cr from both target cells was less that 20%.

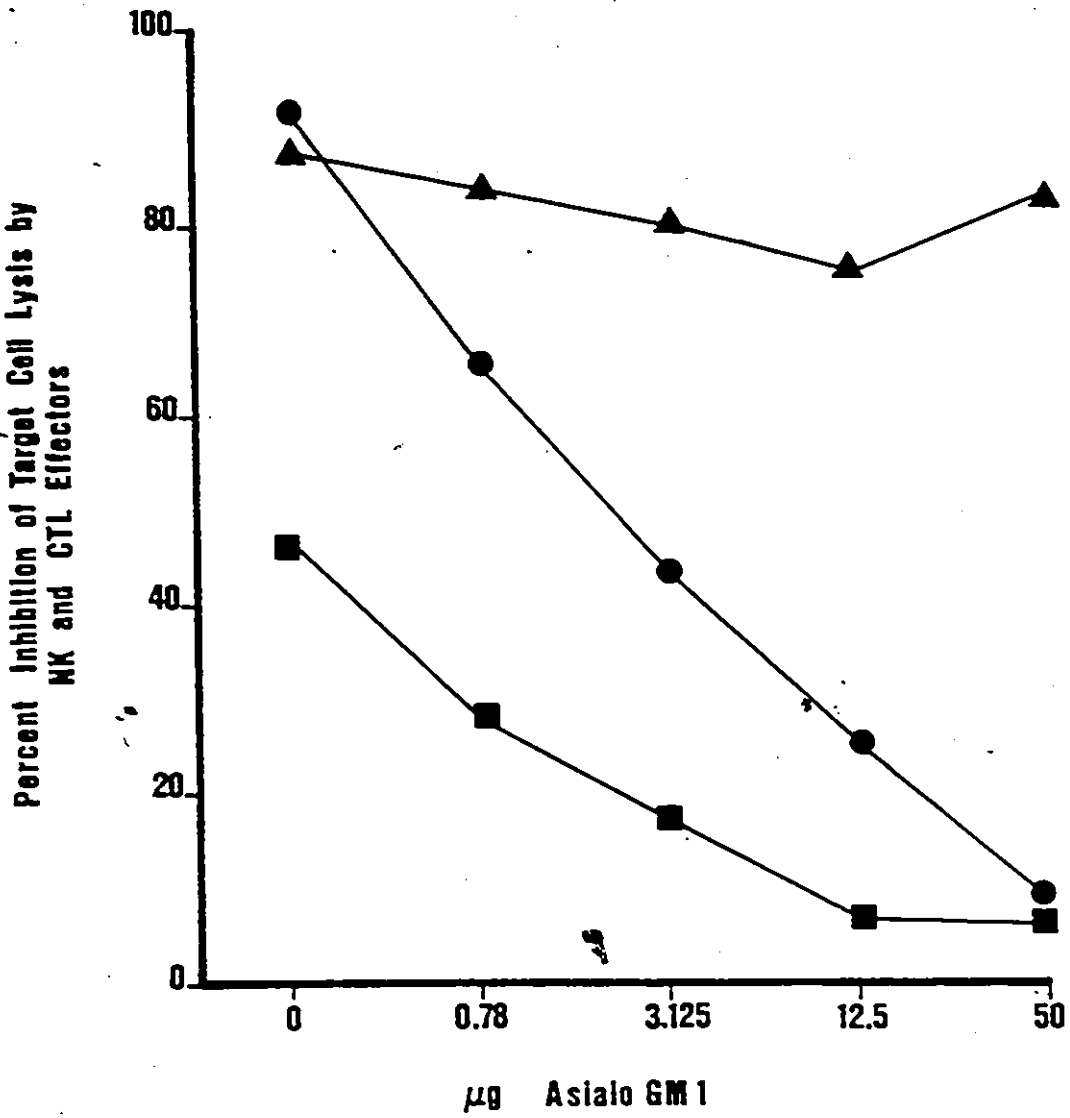

Percent Inhibition of Target Cell Lysis by NK and CTL Effectors
100
80
60
40
20
0
0
0.78
3.125
12.5
50
μg Asialo GM 1

TABLE 11

TITRATION OF ANTI-asGM1 ANTISERUM ON PV-SPECIFIC AND ALLOSPECIFIC CTL[a]

Reciprocal Dilution of Anti-asGM1[b]	CTL Response In Mice Immunized With:			
	PV	(% reduction)	P815	(%reduction)
-	66[c]	(0)[d]	24	(0)
25	12	(82)	1	(96)
100	26	(61)	6	(75)
400	53	(20)	20	(17)
1600	59	(10)	25	(0)

[a] C57Bl/6 mice were immunized i.v. with 6×10^7 pfu of PV or i.p. with 3×10^7 P815 cells. Spleen cells were removed 7 days after PV infection or 12 days after immunization with P815 cells and were assayed for cytotoxicity against ^{51}Cr-labelled PAK-PV or P815 target cells, respectively.

[b] Spleen cells were treated with serial 4-fold dilutions of anti-asGM1 and complement. Treatment of spleen cells with complement alone did not reduce cytotoxicity.

[c] Values represent percent specific ^{51}Cr release at an effector:target cell ratio of 40:1. Spontaneous release of ^{51}Cr from target cells did not exceed 20 percent. PV-specific and allospecific CTL did not cause significant ^{51}Cr release from uninfected PAK cells.

[d] Percent reduction in cytolysis was calculated by:

$$100 - \frac{\text{percent specific } ^{51}\text{Cr release (treated)}}{\text{percent specific } ^{51}\text{Cr release (untreated)}} \times 100$$

observation that PV-specific and allospecific CTL have similar sensitivities to treatment with the antiserum and complement suggests that they express equivalent amounts of asGM1.

3.4 Effect of CY on primary and secondary CTL and NK responses

CY was administered to C57BL/6 mice at various times before primary or secondary challenge with PV. Spleens were removed 7 days after primary infection or 4 days after secondary infection and were assayed for CTL activity against virus-infected and uninfected syngeneic target cells. The results are shown in figure 5. CY (200 mg/kg) given before primary infection with virus markedly reduced CTL responses when compared with those observed in the spleens of virus-infected, untreated control mice. In contrast, administration of CY 18, 24, or 36 hours before secondary challenge did not markedly inhibit CTL responses (figure 5). Both responses were inhibited when the drug was given 9 hours before or at the time of virus infection (figure 5).

The effect of CY on the NK response after primary infection with PV is shown in figure 6. Administration of CY 18 hours before infection resulted in a delay in the generation of virus-induced NK activity. NK activity peaked 7 days after primary infection in CY-treated mice as compared with day 4 in untreated, virus-infected controls (figure 6A). The spleen cells mediating NK activity in CY-pretreated mice were Thy-1^{+ve} and Lyt-2^{-ve} (table 12). Treatment of immune mice with CY 18 hours before secondary challenge did not alter the time of appearance or the magnitude of virus-augmented NK activity (data not shown).

The observed delay in the NK response in CY-treated mice after

Figure 5. Effect of CY on CTL responses to PV. CY was administered at various times before the primary (○) or secondary (●) infection of C57Bl/6 mice with PV. Spleen cell suspensions were prepared as described in Materials and Methods and were tested for cytolytic activity against PV-infected MC57G target cells. The responses in untreated mice after primary (▩) and secondary (▥) PV infection are shown for comparison. The lymphocyte:target cell ratio is 40:1. Spontaneous ^{51}Cr release from PV-infected target cells was less than 15%.

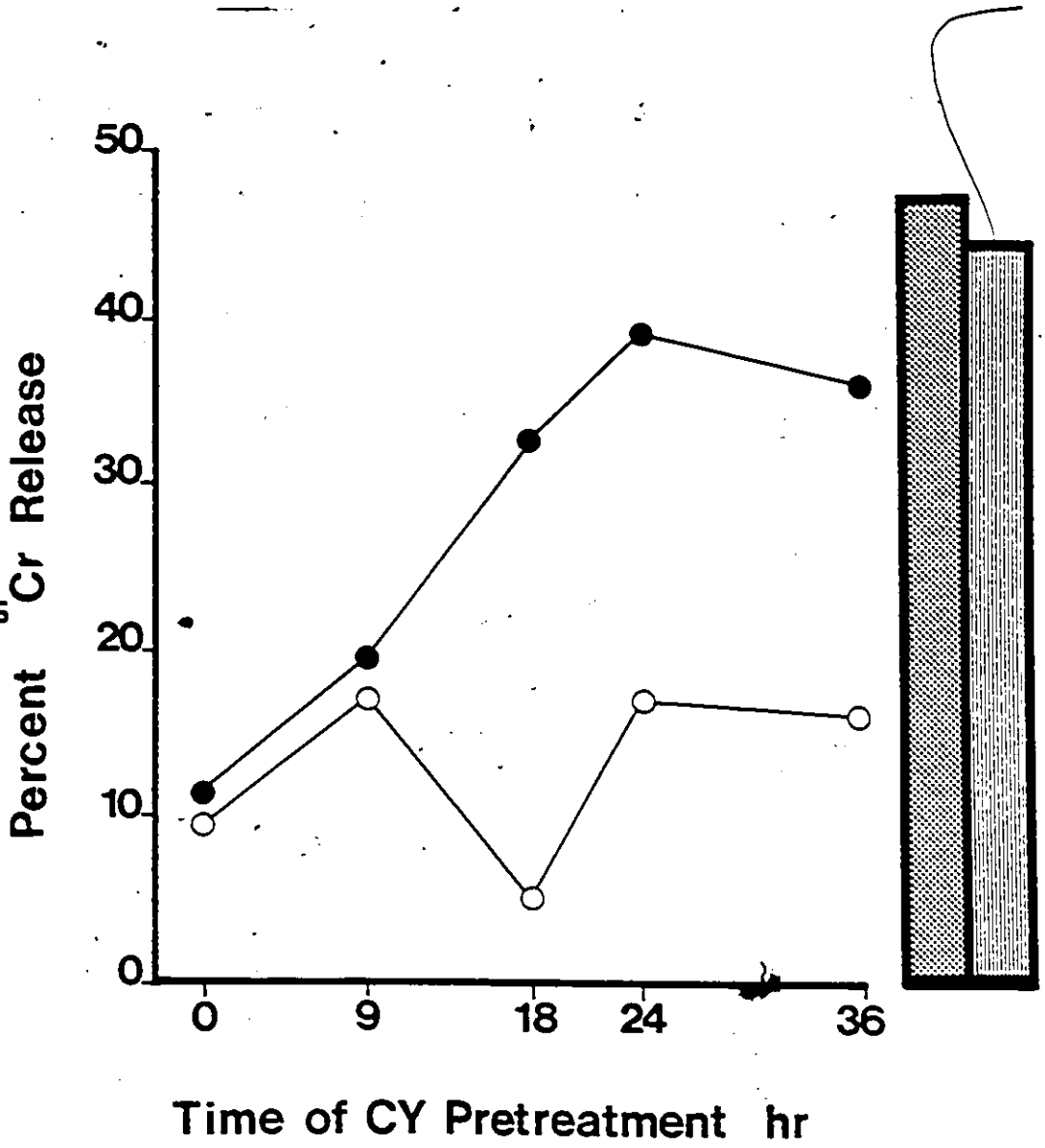

50
40
30
20
10
0
Percent ^{51}Cr Release
0
9
18
24
36
Time of CY Pretreatment hr

Figure 6. Effect of CY on primary NK and CTL responses to PV. C57Bl/6 mice were treated with CY 18 hr before infection with PV. CY- treated (□) and untreated (●) mice were infected with PV at various times before sacrifice . Spleen cells were assayed for cytolytic activity against (A) YAC-1 or (B) PV-infected MC57G target cells. The lymphocyte :target cell ratio was 40:1. Spontaneous ^{51}Cr release from target cells was less than 20%. Reactivity against uninfected MC57G cells was less than 15%.

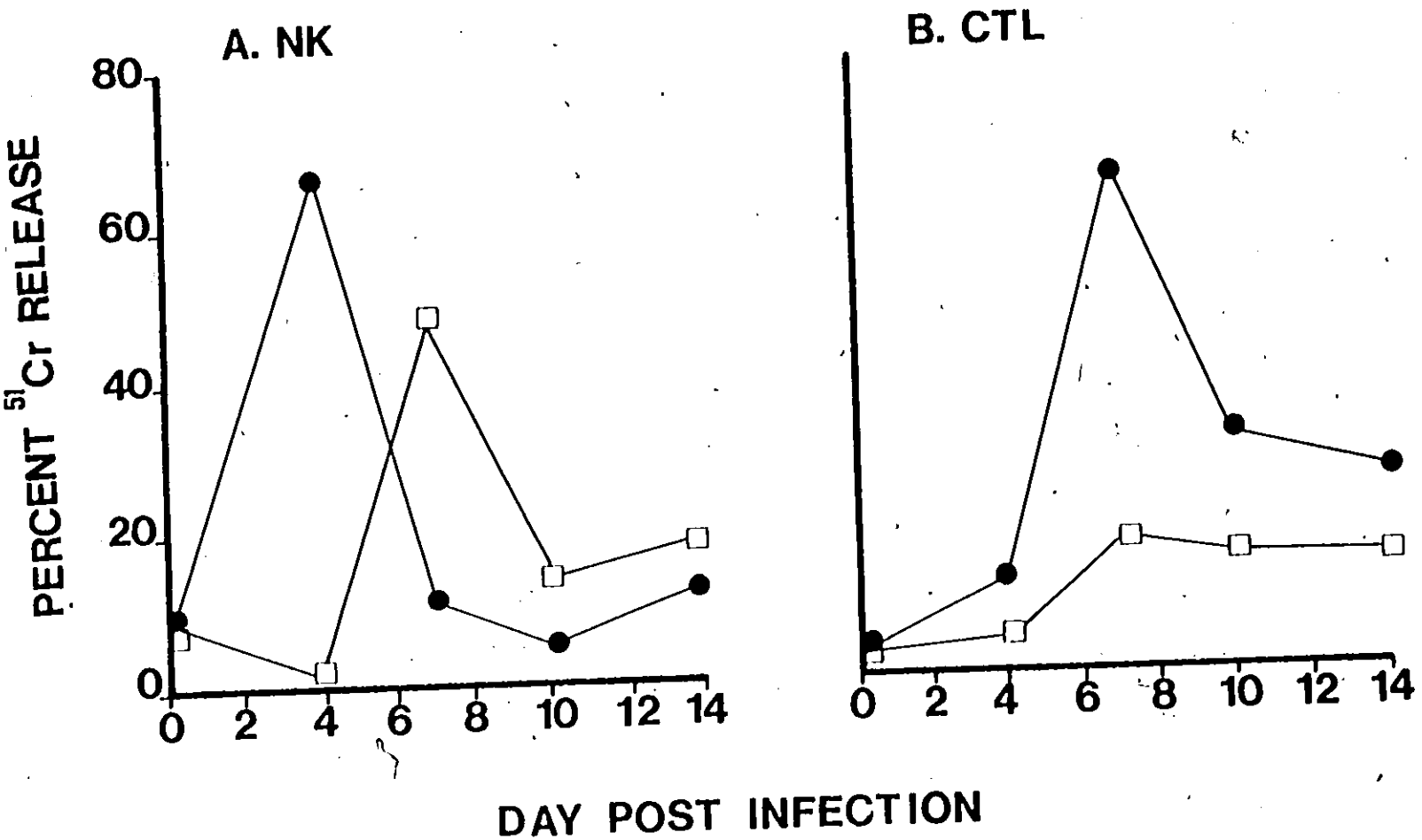

A. NK
B. CTL
PERCENT ^{51}Cr RELEASE
80
60
40
20
0
0 2 4 6 8 10 12 14
0 2 4 6 8 10 12 14
DAY POST INFECTION

TABLE 12

ACQUISITION OF THY-1 AND LYT-2 ANTIGENS BY NK CELLS IN CY-TREATED, PV-INFECTED MICE[a]

Spleen Cells Treated With:		Effector: Target Cell Ratio	Day Post Infection:			
Antibody	Complement		5	6	7	9
-	-	40:1	10[b]	19	44	18
		12:1	2	12	25	11
		4:1	<1	2	9	2
Anti-THY-1	+	40:1	4	6	6	2
		12:1	<1	1	1	<1
		4:1	<1	<1	<1	<1
Anti-LYT-2	+	40:1	11	22	38	17
		12:1	4	9	17	3
		4:1	<1	1	3	<1

[a]C57Bl/6 mice were treated with CY (200 mg/kg) 18 hr before primary i.v. infection with PV. Spleen cells were removed from mice at various times after infection and tested for NK activity against YAC-1 target cells. In some cases spleen cells were treated with monoclonal antibodies to THY-1 or LYT-2 surface antigens and complement prior to assay for cytotoxic activity. Treatment with complement alone did not cause a reduction in NK activity.

[b]Percent specific ^{51}Cr release from YAC-1 target cells in a 4 hr assay. Spontaneous release of ^{51}Cr was less 12 percent.

primary PV infection suggested that the CTL response might be delayed in a similar manner. However, as shown in figure 6b, CTL activity was not generated in CY-treated mice for as long as 14 days after primary infection. These observations indicate that in contrast to primary cell-mediated immune responses, the generation of secondary NK and CTL responses in vivo appears to have a reduced requirement for cell division.

As shown in figure 6, pretreatment with CY delayed the appearance of virus-induced NK activity and abrogated the generation of H-2 restricted, virus-specific CTL. This observation permitted an examination of the effect of priming the NK response on the generation and kinetics of secondary cytotoxic responses. Eighteen hours before primary infection the mice were injected with CY, and five weeks later they were rechallenged with PV. One day after secondary infection an augmented NK response was detected that quickly returned to background levels (table 13). This response was comparable to that observed on day 1 after secondary infection of the untreated mice. In contrast, the mice that had received CY before primary infection with PV did not generate a marked H-2 restricted, virus-specific CTL response after rechallenge (table 13). Rather, a low but significant cytotoxic response against syngeneic virus-infected cells was detected 1 day after reinfection, and this activity remained constant through day 7. Since this low level cytotoxicity was H-2 restricted and virus-specific (table 13), and could be abrogated by pretreatment of effector cells with anti-Thy-1.2 or anti-Lyt-2.2 and complement (data not shown), it appeared to be mediated by CTL.

These results demonstrate that after CY treatment, a delayed

TABLE 13

EFFECT OF CY PRETREATMENT ON THE GENERATION OF SECONDARY NK AND CTL RESPONSES TO PV[a]

Days Post Secondary Infection	CY	Lymphocyte: Target Cell Ratio	Percent Specific ^{51}Cr Release[b]				
			YAC-1	MC57G			L929
				uninfected	PV	LCMV	PV
1	-	40:1	20	7	29	16	<1
		12:1	13	<1	17	5	<1
		4:1	4	2	10	4	<1
4	-	40:1	15	2	37	4	<1
		12:1	9	<1	21	3	<1
		4:1	4	3	19	4	<1
1	+	40:1	36	8	18	10	<1
		12:1	11	<1	9	4	<1
		4:1	9	3	5	5	<1
4	+	40:1	16	<1	15	6	<1
		12:1	7	2	10	1	<1
		4:1	4	3	7	6	<1
7	+	40:1	18	4	18	4	<1
		12:1	7	1	13	1	<1
		4:1	4	2	10	4	<1

[a]CY (200 mg/kg) was administered to C57Bl/6 mice 18 hr before primary infection. Five weeks later the mice were rechallenged with PV at various times before testing. Effector spleen cells taken 1 and 4 days after secondary infection were included as positive controls for NK and CTL cytotoxicity, respectively.

[b]Assay time 4 hr for YAC-1 and 7.5 hr for MC57G and L929 targets; spontaneous release <25%.

virus-induced NK response can occur in the absence of a CTL response. Furthermore, rechallenge of these NK-primed mice with virus results in the rapid generation of a memory NK response that is accompanied by persistent low level augmentation of CTL activity.

3.5 Effect of CY on virus titers in PV infected mice

It was possible that CY treatment before primary PV infection resulted in a persistent infection that prevented the generation of memory CTL upon reexposure to virus. Accordingly, C57BL/6 mice were treated with CY 18 hr before PV infection, and virus titers were determined in the spleen, brain, lymph nodes, and serum at various times afterwards. Results shown in table 14 demonstrate that mice not previously treated with CY cleared PV from serum and spleen by 4 days postinfection. In contrast, virus titers in the spleen and serum of CY-treated mice peaked at 4 days after infection and subsided thereafter (table 14). Virus (2.6 pfu/gm) was detected in the lymph nodes of CY-treated mice 35 days after infection, whereas no virus was detected in lymph nodes of untreated mice at any time (data not shown). In contrast to these results, treatment of mice with CY 18 hr before infection with LCMV resulted in establishment of a persistent infection. Virus titers (expressed as $\log_{10}$ pfu per gram of solid tissue or per milliliter of serum) in the spleen, serum, and brain were 5.7, 5.3, and 5.0, respectively, 35 days after infection. These observations suggest that CY-treated mice cleared PV from the serum and most tissues, except lymph nodes, a few days later than untreated mice and were not persistently infected as were CY-treated, LCMV-infected mice.

TABLE 14

EFFECT OF CY ON VIRUS TITERS IN MICE INFECTED WITH PV

Organ	CY[a]	Virus Titer[b] At Day After Infection				
		Day 1	Day4	Day8	Day20	Day35
Spleen	-	6.0	<1	<1	<1	<1
	+	5.7	7.0	<1	<1	<1
Serum	-	1.8	<1	<1	<1	<1
	+	2.2	3.4	ND[c]	<1	<1

[a] CY (200 mg/kg) was administered by i.p. injection to C57Bl/6 mice 18 hr before infection with PV.

[b] Log_{10} pfu of PV per milliliter of serum or per gram of spleen.

[c] ND = not done.

3.6 Restoration of PV-specific memory CTL responses in CY-pretreated mice

3.6.1 Effect of co-infection on memory anti-PV CTL responses.

It was reported (Zinkernagel and Doherty, 1974b) that T cell tolerance observed in LCMV carrier mice could be broken by innoculation of allogeneic normal lymphocytes. In light of this, it was reasoned that co-infection with PV and a distinct virus might restore memory anti-PV CTL responses in CY-treated, PV-primed mice. Mice were treated with CY, infected with PV, and 5 wk later were rechallenged with PV, LCMV, or both viruses together. Spleens were removed at various intervals and were tested for CTL activity. Mice infected with PV or LCMV alone did not generate detectable PV-specific CTL (figure 7). However, mice co-infected with both PV and LCMV generated a strong cytotoxic response that peaked 4 days later and declined thereafter (figure 7). This peak of cytotoxic activity appeared to be mediated by CTL as syngeneic PV-infected target cells were lysed but LCMV-infected target cells were not (table 15,expt. 1) Thus, co-infection with PV and LCMV appears to restore memory anti-PV CTL responses in CY-treated mice.

To determine whether coinfection with viruses other than LCMV could restore memory responses, CY-treated, PV-infected mice were rechallenged 5 wk later PV and TV, or PV and VSV. Infection with PV, TV, or VSV alone did not elicit memory anti-PV CTL responses (table 15). However, coinfection with PV and TV resulted in a PV-specific memory CTL response (table 15, expt.2). Coinfection with PV and the unrelated rhabdovirus VSV resulted in only a slight augmentation of PV-specific CTL activity 4 days later (table 15, expt.3). Thus,

Figure 7. Coinfection restores memory PV-specific CTL in CY-treated, PV-primed mice. Mice were treated with CY (200 mg/kg) 18 hr before infection with PV. Five weeks later mice were rechallenged with PV (○) or LCMV (■) alone, or were co-infected with PV and LCMV (●) at various times before assay for CTL activity against PV-infected PAK 11 target cells. Assay duration was 7.5 hr and spontaneous release was less than 25%. Lymphocyte: target cell ratio was 40:1. Spleen cells from each effector group failed to cause significant lysis of uninfected or LCMV-infected PAK 11 target cells. (data not shown).

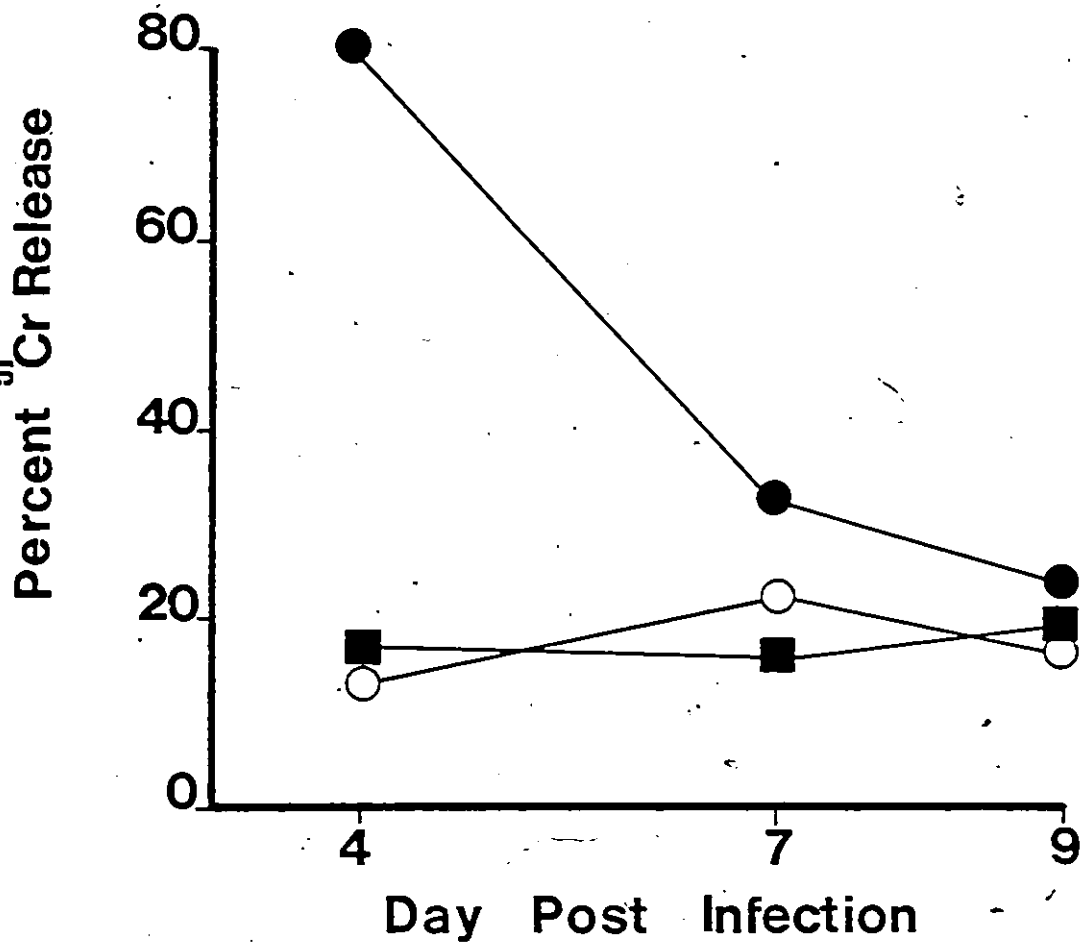

Percent ^{51}Cr Release
80
60
40
20
0
4
7
9
Day Post Infection

TABLE 15

COINFECTION RESTORES MEMORY ANTI-PV CTL RESPONSES IN CY-TREATED VIRUS-PRIMED MICE[a]

Experiment	Secondary Challenge: PV	Secondary Challenge: Coinfecting Virus	Effector: Target Cell Ratio	Percent Specific ^{51}Cr Release[b] from MC57G Target Cells: uninfected	PV	TV	LCMV
1	+	-	40:1	3	16	ND[c]	<1
			12:1	<1	4	ND	6
			4:1	<1	3	ND	<1
	-	LCMV	40:1	8	13	ND	12
			12:1	<1	7	ND	<1
			4:1	3	8	ND	4
	+	LCMV	40:1	16	80	ND	16
			12:1	8	57	ND	10
			4:1	3	29	ND	4
2	+	-	40:1	12	24	10	ND
			12:1	3	12	7	ND
			4:1	5	11	4	ND
	+	TV	40:1	21	65	18	ND
			12:1	17	38	12	ND
			4:1	12	23	5	ND
3	+	-	40:1	2	33	ND	ND
			12:1	<1	20	ND	ND
			4:1	<1	3	ND	ND
	-	VSV	40:1	1	<1	ND	ND
			12:1	2	4	ND	ND
			4:1	3	<1	ND	ND
	+	VSV	40:1	8	43	ND	ND
			12:1	3	25	ND	ND
			4:1	1	7	ND	ND

[a] C57B1/6 mice were injected with CY followed 18 hr later with PV. Five weeks later were rechallenged with PV with or without coinfection. Spleen cells from these mice were tested for cytotoxicity 4 days after rechallenge.

[b] Assay time was 7.5 hr, spontaneous release was less than 25 percent.

[c] ND = not done.

coinfection with PV and other arenaviruses (TV or LCMV) was able to restore memory PV-specific CTL responses in vivo and was more efficient than coinfection with VSV.

3.6.2 IL 2-containing supernatants reconstitute memory CTL responses

It seemed possible that coinfection with LCMV or TV resulted in the generation of helper factors (i.e. lymphokines) necessary to elicit memory anti-PV CTL responses in CY-treated mice. To test this possibility, CY-treated, PV-primed mice were rechallenged 5 wk later with PV and lymphokines were provided by i.p. injection. Two milliliters of rat Con A supernatant or supernatant from MLA-144 cells were administered at the same time as virus rechallenge and then again at daily intervals for 3 days. Cytotoxic activity in the spleen was assessed on day 4 after rechallenge. As shown in table 16, reinfection of CY-treated mice with PV alone did not result in anti-viral CTL activity. However, mice treated with both PV and IL 2-containing supernatant efficiently lysed PV-infected histocompatible target cells (table 16). NK-sensitive YAC-1 or TV-infected target cells were not killed (table 16), indicating that cytotoxicity was mediated by CTL and not NK cells. Mice that received Con A supernatant alone without reinfection with PV did not generate virus-specific CTL (table 16). Thus, administration of IL2-containing supernatants in vivo permitted the generation of memory anti-PV CTL in CY-treated, PV-primed mice after virus rechallenge.

A similar restoration of anti-PV CTL activity occurred when IL 2-containing supernatants were added to in vitro cultures of responder spleen cells taken from CY-treated, PV-primed mice. Spleen cells from these mice did not generate significant levels of

anti-viral cytotoxicity when restimulated with homologous virus in vitro (table 17). However, the addition of rat Con A supernatant or MLA 144 supernatant (10% v/v) to culture medium restored cytotoxic activity against PV-infected target cells to control levels (table 17). The addition of IL 2-containing supernatants to cultures containing uninfected macrophages did not elicit a virus-specific cytotoxic response (table 17).

3.6.3 Generation of memory CTL by purified IL 2

Since both rat Con A supernatant and MLA 144 supernatant contain IL 2, it was important to determine whether purified IL 2 alone could restore CTL responsiveness to spleen cells from CY-treated, PV-primed mice. Figure 8 shows that the addition of highly purified IL 2 to in vitro secondary cultures resulted in a dose dependent restoration of anti-PV CTL activity. Further, PV-infected histoincompatible (L929) target cells were not lysed (figure 8), which suggested that cytotoxic activity was mediated by H-2 restricted CTL. These results indicate that highly purified IL 2 is necessary and sufficient to reconstitute memory anti-viral CTL responsiveness to spleen cells from CY-treated mice.

3.7 Frequency of PV-specific CTL precursors in CY-treated and untreated mice

The ability of coinfection or IL 2-containing supernatants to restore memory anti-PV CTL responses in CY-treated mice suggested that CY treatment affects helper T cells and not CTL precursors. To examine this contention, the frequency of CTLp to PV was determined in CY-treated and untreated mice by limiting dilution analysis. Graded

TABLE 16

ABILITY OF IL 2-CONTAINING SUPERNATANTS TO RESTORE MEMORY CTL RESPONSES IN CY-TREATED PV-PRIMED MICE[a]

Primary Immunization		Secondary Immunization		Effector to Target cell ratio	Percent Specific ^{51}Cr Release From Target Cells[c]			
					PAR			
CY	PV	PV	Supernatant[b]		uninfected	PV	TAC	YAC-1
-	+	+	-	40:1	10	54	10	17
				12:1	2	37	6	9
				4:1	<1	17	2	6
+	+	-	-	40:1	3	9	1	14
				12:1	<1	3	3	5
				4:1	6	5	3	4
+	+	+	-	40:1	3	6	9	9
				12:1	4	7	ND[d]	6
				4:1	6	4	ND	3
+	+	+	MLA-144	40:1	9	60	7	22
				12:1	5	41	3	11
				4:1	11	20	4	6
+	+	+	Rat ConA	40:1	15	35	6	16
				12:1	12	18	4	7
				4:1	12	8	1	4
+	+	-	Rat ConA	40:1	11	14	4	17
				12:1	14	12	5	7
				4:1	13	4	5	3

[a] Five weeks after CY- PV treatment, C57Bl/6 mice were rechallenged with PV and injected i.p. with IL 2-containing supernatants. Spleen cells were tested for cytotoxicity 4 days after rechallenge.

[b] Two milliliters of each supernatant were injected i.p. on the day of and for 3 days after rechallenge with PV.

[c] Assay time 7.5 hr, spontaneous release <25%.

[d] ND = not done.

TABLE 17

GENERATION OF CTL IN VITRO AFTER ADDITION OF IL 2-CONTAINING SUPERNATANTS TO SPLEEN CELLS FROM CY-TREATED, PV-PRIMED MICE

Expt.	Primary Immunization[a]		Secondary Culture[b]		Percent Specific ^{51}Cr Release From Target Cells[c]		
					PAK		L929
	CY	PV	Macrophages Infected With	Supernatant	Uninfected	PV	PV
1	-	+	PV	---	18	75	ND[d]
	+	+	PV	---	5	12	ND
	+	+	PV	Rat Con A	25	58	ND
	+	+	--	Rat Con A	32	19	ND
2	-	+	PV	--	22	75	ND
	+	+	PV	--	6	32	3
	+	+	PV	MLA-144	17	57	16
	+	+	--	MLA-144	34	26	4

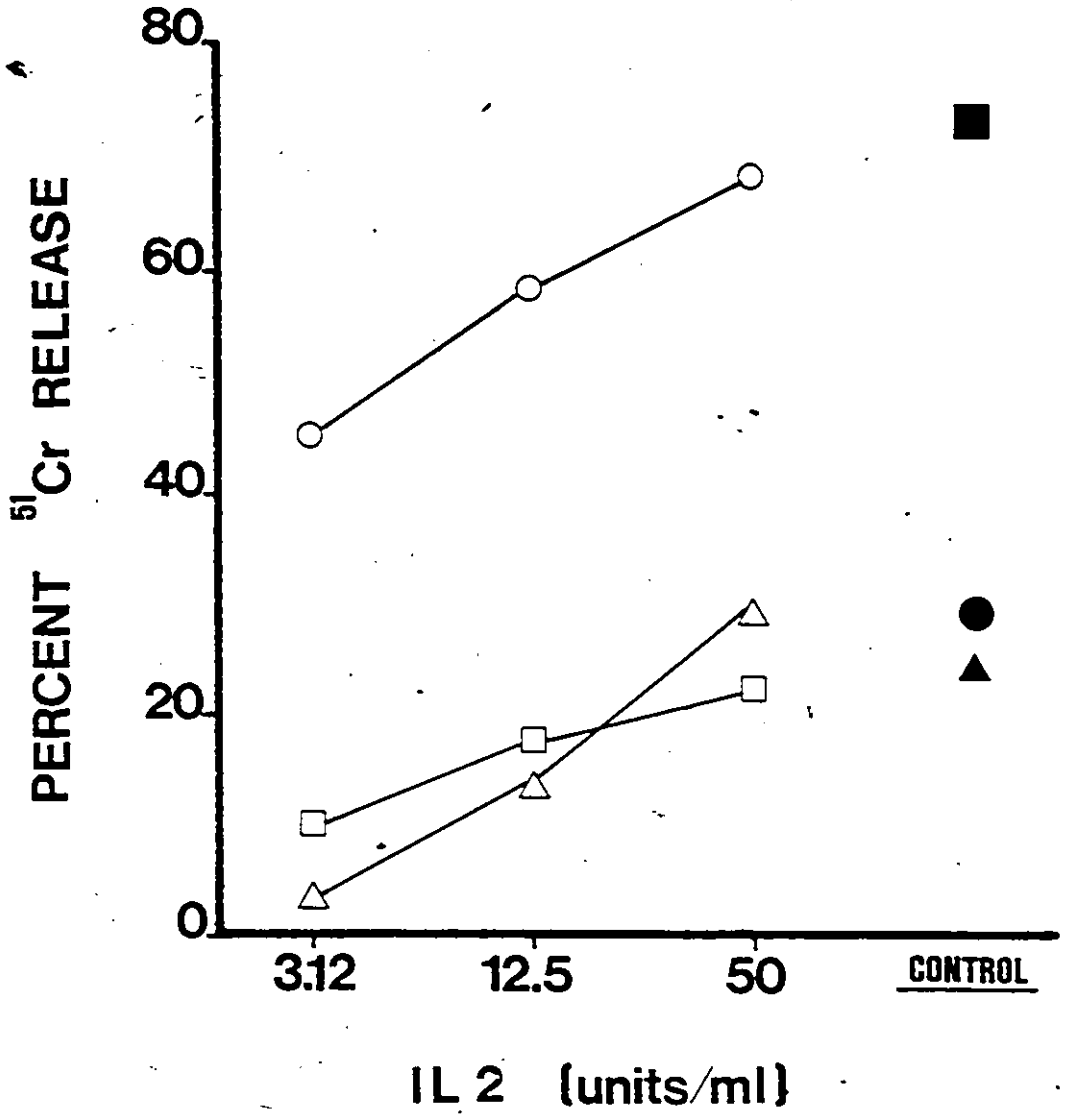
[a] CY (200 mg/kg) was administered to mice 18 hr before primary infection with PV

[b] Five weeks later, spleen cells were removed and incubated in vitro with irradiated (1500R) uninfected or infected macrophages. IL 2-containing supernatants (10% v/v) were added at the initiation of culture. Cultures were assayed for cytotoxicity 5 days later. Effector cell concentrations were not readjusted before being assayed for cytotoxicity.

[c] Assay time was 7.5 hr, spontaneous release was less than 25 percent.

[d] ND = not done.

Figure 8. Purified IL 2 restores anti-PV CTL responses in vitro. C57Bl/6 ($H\text{-}2^b$) mice were treated with CY (200 mg/kg) 18 hr before infection with PV. Five weeks later spleen cells were removed and restimulated in vitro with irradiated PV-infected macrophages as described in Materials and Methods. Highly purified IL 2 was added to the culture supernatant at the indicated concentration; 5 days later cultures were tested for CTL activity against syngeneic uninfected (□——□) or PV infected (○——○) PAK 11 ($H\text{-}2^b$) or allogeneic L929 PV ($H\text{-}2^k$) infected (△——△) target cells. Controls included spleen cells from untreated PV- primed mice that were restimulated with PV-infected (■) or uninfected (●) syngeneic macrophages and were tested for cytotoxic activity against PAK 11 -PV. Spleen cells from CY-treated PV-infected mice were also restimulated in vitro with PV-infected syngeneic macrophages in the absence of IL 2 (▲). Effector cell concentration was not adjusted before assay. Assay duration was 7.5 hr. Spontaneous release was less that 25%.


80
60
40
20
0
PERCENT ^{51}Cr RELEASE
3.12
12.5
50
CONTROL
IL 2 (units/ml)

numbers of responder spleen cells from CY-treated and untreated PV-primed mice were cultured in microtiter wells with PV-infected stimulator cells. Lectin-free human T cell growth factor was added to culture medium as a source of IL-2. At a 5 percent (v/v) concentration, this growth factor promoted the generation of PV-specific CTL activity while minimizing the production of antigen non-specific lymphokine activated killer cells that are capable of lysing LCMV-infected target cells (table 18). Accordingly, this concentration of supernatant was used as a source of help in all subsequent limiting dilution assays.

Limiting dilution analysis indicates that the number of PV-specific CTLp in mice not treated with CY is approximately 50-fold higher than that in CY-pretreated, PV-primed mice (i.e. 1/64,953 vs $1/2.812 \times 10^6$) (figure 9). Thus, it appears that CY pretreatment affects both T helper cells and anti-viral CTLp.

TABLE 18

TITRATION OF HUMAN IL 2-CONTAINING SUPERNATANTS ON PV-SPECIFIC CTL_P[a]

Percent of IL 2-containing supernatant in culture medium:	Virus-infected targets added to wells (number/well)			
	PAK-PV (2.5×10^4)	PAK-PV (1.25×10^4)	PAK-PV (0.75×10^4)	PAK-LCMV (2.5×10^4)
20	12[b]	11	10	4
10	12	12	12	3
5	12	12	12	2
2.5	12	12	8	1

[a]C57Bl/6 mice were infected i.v. with 7×10^6 pfu of PV. Six weeks later mice were sacrificed and 30×10^4 spleen cells were cultured in flat bottomed microtiter wells with 1×10^5 virus-infected stimulator cells in 200 ul of culture medium containing various concentrations of human IL 2 supernatant. Five days later varying numbers of PAK target cells infected with LCMV or PV were added to individual culture wells in a volume of 50 ul. 7.5 hours the assay was terminated and the amount of ^{51}Cr released into the culture supernatant was determined. Spontaneous release of ^{51}Cr was determined by incubating target cells with stimulator cells alone. Wells were scored as positive if the counts released were greater than or equal to 2 SD above the spontaneous release.

[b]Number of responding wells (out of 12).

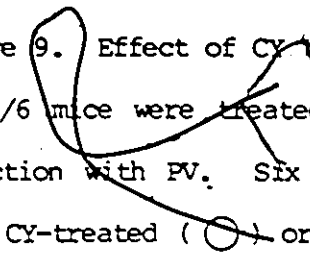
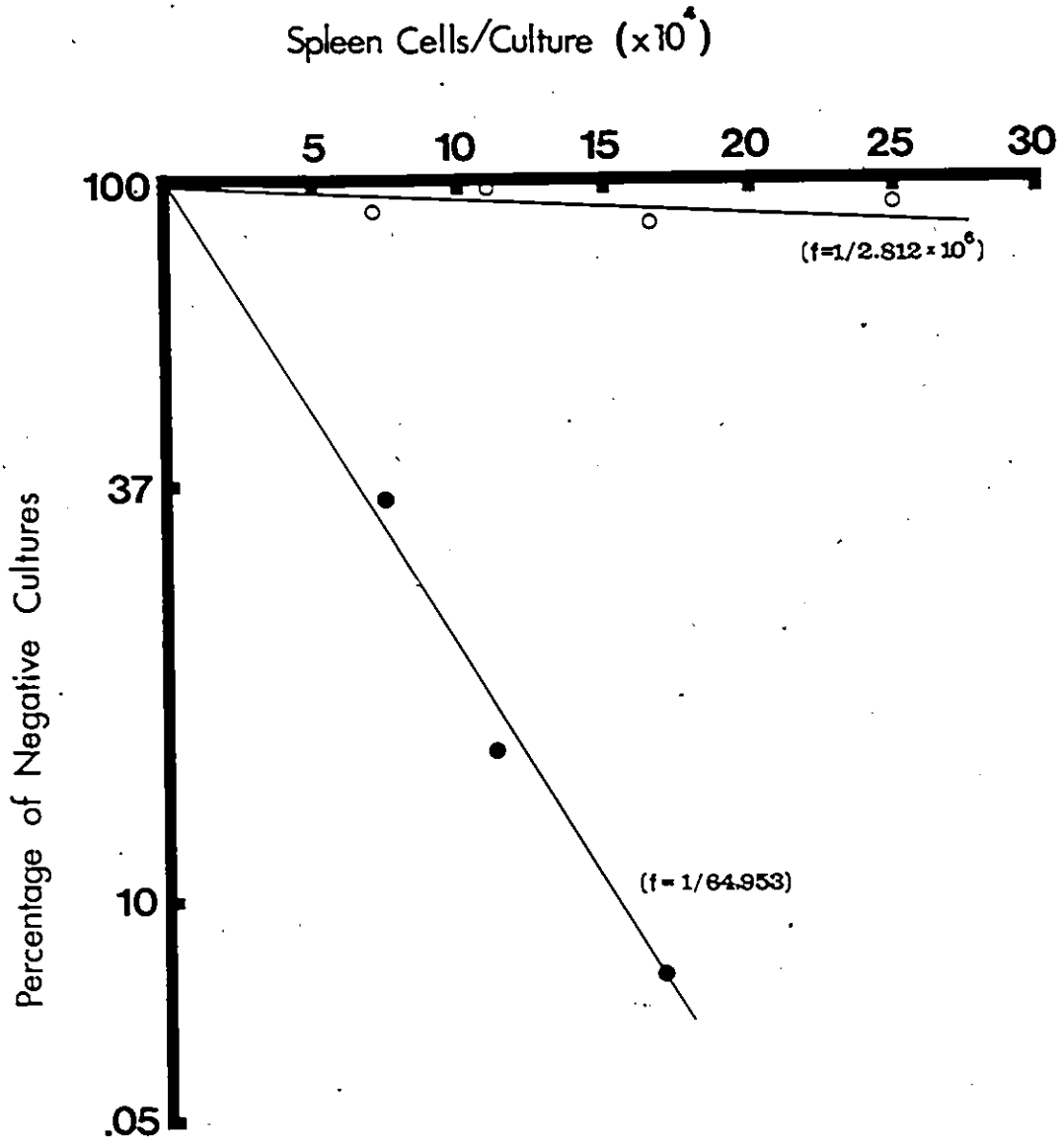


Figure 9. Effect of CY treatment on CTL precursor frequency. C57Bl/6 mice were treated with CY (200 mg/kg) 18 hr before infection with PV. Six weeks after infection, spleen cells from CY-treated (○) or untreated (●) mice were cultured with stimulator cells for 5 days and then assayed for cytotoxicity as outlined in Materials and Methods. Spontaneous release of ^{51}Cr was determined by incubating target cells with stimulator cells alone. Wells were scored as positive for cytotoxicity if the counts released were greater than or equal to 2 SD above spontaneous release. Each point represents the result of 24 replicate cultures at the indicated number of responder spleen cells. The Chi-squared value (which represents the correlation co-efficient of the line) was 0.829 and 4.7 for untreated and CY-treated mice, respectively. The 95% confidence interval for untreated mice is 1/49,462 to 1/85,295. The 95% confidence interval for CY-treated mice is $1/1.196 \times 10^6$ to $1/6.611 \times 10^6$.


Spleen Cells/Culture (x10⁴)
5
10
15
20
25
30
100
37
10
.05
Percentage of Negative Cultures
(f=1/2.812 x 10⁶)
(f = 1/64.953)

CHAPTER FOUR

DISCUSSION

The present studies were undertaken to determine whether inbred strains of mice differ in their susceptibility to PV infection. In addition, cell-mediated immune responses to PV were assessed in mice of various haplotypes, and the relationship between NK and CTL effectors was examined. Attention was focused on the hypothesis that enhanced NK activity detected in the spleens of mice after PV infection is mediated by CTLp that are maturing to become H-2 restricted, virus-specific CTL.

4.1 Susceptibility of inbred mice to infection with prototype (strain AN3739) or spleen-passaged PV strains.

Inbred strains of adult mice were found to be uniformly resistant to lethal infection by PV, thus confirming earlier observations by others (Trapido and Sanmartin, 1971; Buchmeier, 1976). Furthermore, attempts to generate virulent strains of PV by repeated forced passage of the prototype virus through the spleens of newborn mice proved unsuccessful. Prototype PV that was passaged through newborn B10.Br mice was not lethal for adult mice of the same strain. In addition, the passaged and prototype viruses replicated equally well in the spleens of adult B10.Br mice. It is not clear why PV did not adapt to mice. One possibility is that the virus is genetically stable *in vivo*, so that virulent variants could not be selected by forced passage through the spleens of newborn hosts. This is unlikely, however, as PV variants that are lethal for inbred guinea pigs have been generated using a similar passage scheme to the one described here (Jahrling *et al.*, 1981). The tissue tropism and pathogenicity of other arenaviruses (notably LCMV and Junin virus) can

also be altered by passage of wild-type virus through mice or guinea pigs (Lehmann-Grube et al., 1982a; Laguens et al., 1983).

An alternate explanation is that PV variants did arise during the passage procedure, but that a rapid and efficient host response to the virus allowed adult B10.Br mice to cope successfully with infection. However, it is also possible that the target cell supporting the replication of passaged PV in the newborn mouse spleen disappears as the mouse matures, the result of normal differentiation. Mouse spleen cells show such an age-dependent susceptibility to infection with LCMV; spleen cells (predominately of the T lineage) can be infected with LCMV shortly after birth, whereas spleen cells in adult mice are considerably more resistant to infection (Popescu et al., 1982; Lehmann-Grube et al., 1983).

4.2 Characterization of cell-mediated immune responses to PV.

All strains of mice tested generated strong cell-mediated immune responses after infection with prototype PV. Inoculation of PV into the footpads of mice elicited a delayed-type hypersensitivity (DTH) response that peaked 7 days later. Additionally, two types of cytotoxic activity could be detected in the spleens of inbred mice after i.v. challenge with PV; enhanced NK activity was found to peak 3-4 days after primary infection, and subsided by day 7 when a PV-specific, H-2 restricted CTL response was found to be maximal. These results confirm earlier observations of augmented NK and CTL activities after challenge of mice with PV (Welsh, 1978; Buchmeier et al., 1980).

Mice of various haplotypes were also shown to generate memory cytotoxic cell responses after secondary in vivo infection with PV.

Augmented NK activity was detected 1 day after reinfection, and this was followed 3 days later by a peak of CTL activity. The generation of a memory CTL response after rechallenge with PV was unexpected; similar efforts to elicit such a response after reinfection in vivo with a variety of other viruses (including ectromelia, influenza, rabies, Sendai, and VSV) have been unsuccessful (Gardner and Blanden, 1976; Schrader and Edelman, 1977; Effros et al., 1977; Wiktor et al., 1977; Rosenthal and Zinkernagel, 1980). Two explanations have been put forward to account for this unresponsiveness. On one hand, it has been argued that neutralizing antibodies elicited after primary infection rapidly eliminate or alter the antigen presentation of reinfecting virus (Zinkernagel and Doherty, 1979). The observation that mice do not produce neutralizing antibodies in response to PV infection, and yet generate memory CTL activity when rechallenged with homologous virus, supports this view.. However, Wolcott and co-workers (1982) have demonstrated that reinfection of mice with homologous alphaviruses elicits memory CTL activity even in the presence of neutralizing antibodies. A similar observation has been made by Lehmann-Grube et al. (1981), who demonstrated that neutralizing antibodies produced in response to primary infection with a low dose of LCMV (strain WE) do not interfere with the generation of memory CTL activity in vivo after rechallenge with a high dose of homologous virus. Conversely, mice infected with LCMV (strain E-350) do not generate memory CTL activity when reinfected with the same virus, even though neutralizing antibodies cannot be detected (Lehmann-Grube et al., 1982b). Taken together, these observations suggest that an active suppressor mechanism, and not a neutralizing

antibody response, is responsible for the block in secondary CTL generation to most viruses.

This model system provided a unique opportunity to assess the requirement for cell division in the in vivo generation of primary and secondary CTL responses to PV. CY (an alkylating agent that is thought to inhibit cell division in vivo; Stockman et al., 1978) was found to block the generation of primary CTL activity when administered 18 hr before infection with PV. However, similar treatment prior to secondary challenge of mice with PV did not inhibit the generation of memory CTL activity. Thus, memory CTL are similar to memory B cells in that they appear to have a reduced requirement for cell division upon reactivation in vivo. This view is corroborated by studies showing that in vitro reactivation of primed alloreactive CTL does not require DNA synthesis (MacDonald et al., 1975; Wagner and Rollinghoff, 1976; Senik and Bloom, 1977).

Few attempts have been made to examine secondary virus-induced NK responses. This may not seem unusual inasmuch as a non-specific effector mechanism would not be expected to display antigen-specific memory. Previous studies by Welsh (1978) failed to detect enhanced NK activity in PV or LCMV primed mice following secondary rechallenge with homologous virus. However, in his studies cytotoxicity was examined 3 days after reinfection, when primary NK activity is maximal. The data presented here demonstrate that augmented NK activity peaked 1 day after reinfection with PV, and rapidly subsided to background levels. As was observed for the generation of secondary CTL, memory virus-induced NK could be elicited in mice that were pretreated with CY. The accelerated appearance of

this response, and its apparent lack of requirement for cell division, suggests that PV-primed NK cells possess antigen-specific memory. Virus-specific NK memory could reside in a population of primed NK effectors that respond directly to antigen, or it could reside in a population of antigen-primed T cells capable of producing lymphokines that augment NK activity, such as IL 2 or the interferons.

Welsh (1978) has speculated that virus-induced NK activity is shut down by the development of a T cell dependent immune response, as NK cell activity remains elevated in LCMV-infected nude mice long after it has declined in nude/+ controls. Presumably, T cells could depress the cytolytic activity of activated NK cells by inhibiting synthesis of interferons. Thus, the more rapid appearance of CTL after secondary infection could result in the early shutdown of an NK response that would otherwise peak 4 days (or more) after reinfection. However, the observation that memory NK activity induced in CY-treated, PV-primed mice is accompanied by a persistent, low-level CTL response, and yet still peaks one day after reinfection, argues against this explanation.

4.3 Relationship of PV-induced NK cells to virus-specific CTL.

As noted earlier, a three day interval separated peak NK and CTL activities after primary or secondary immunization with PV, even though memory responses peaked earlier than primary responses. Maintenance of the temporal relationship between NK and CTL generation suggested that augmented NK activity is mediated by activated CTLp that are maturing to become H-2 restricted, virus-specific CTL.

4.3.1 Shared expression of cell surface antigens on NK cells and CTL

If NK cells differentiate to become CTL, then it might be

expected that they would lose certain "NK-specific" cell surface antigens and acquire T cell markers. To approach this question, PV induced NK cells were examined for expression of Thy-1, a pan T cell marker that is absent (or expressed at very low levels) on the surface of endogenous NK cells (Herberman et al., 1977; Pollack et al., 1979; Koo et al., 1980). The results presented here suggest that with time, both primary and secondary PV-induced NK effectors express more Thy-1 antigen, which is consistent with the idea that they are maturing to become H-2 restricted, PV-specific CTL. A similar conclusion was reached by Minato and co-workers (1981), who showed that Thy-1 negative NK cells are precursors to Thy-1 positive cells. This observation is further supported by the finding of increased Thy-1 on activated NK cells after primary infection of mice with LCMV (Herberman et al., 1978; Kiessling et al., 1980) and on AK cells generated in vitro (Shain et al., 1982; Karre et al., 1983).

It has been reported that antibodies to the glycolipid asGM1 (gangliotetraosylceramide) react preferentially with NK cells and not other lymphocyte subsets (Kasai et al., 1980a; Young et al., 1980; Schwarting and Summers, 1980). This reactivity was initially discovered by absorption studies using an antiserum against brain-associated T cell antigen (Thy-1), which was cytotoxic for both NK and T cells (Habu et al., 1979). Absorption of the antiserum with asGM1 removed NK cell reactivity but it remained cytotoxic for T cells. A polyclonal rabbit antiserum to purified asGM1 has been widely used to deplete NK cell activity both in vitro (Kasai et al., 1980a; Young et al., 1980) and in vivo (Habu et al., 1981). When the specificity of the antibody was studied using immunofluorescence

techniques, about 30 percent of Thy-1 positive spleen cells also reacted with anti-asGM1 antibodies (Stein et al., 1978). However, no reactivity could be detected against MLC-generated CTL, thymocytes, Con A reactive T cells, or carrier-specific T helper cells using a dilution of anti-asGM1 that eliminated endogenous NK activity in complement-dependent cytotoxicity assays (Kasai et al., 1980a; Young et al., 1980). From this data one might predict that if NK cells are pre-CTL, then PV-primed CTLp should express asGM1. The results presented here suggest that this is the case; the in vitro generation of PV-specific CTL was prevented if PV-primed responder cells were pretreated with anti-asGM1 and complement prior to secondary in vitro restimulation with antigen. Similar results have been reported by Beck and co-workers (1982) for allospecific CTLp. It is possible that treatment of responder spleen cells with anti-asGM1 and complement eliminated helper cells necessary for the generation of CTL rather than CTLp themselves. Indeed, other investigators have shown that NK cells produce a number of lymphokines and have an accessory role in the generation of allospecific and virus-specific CTL (Kasahara, 1983; Burlington et al., 1984; Scala et al., 1984; Pistoia et al., 1985; Scala et al., 1985; Suzuki et al., 1985). Suzuki and coworkers (1985) showed that spleen cells from mice given a single injection of anti-asGM1 do not give rise to allospecific CTL when stimulated with allogeneic cells in vitro. This unresponsiveness could be reversed if highly purified IL 2 or alpha IFN was added to in vitro cultures, suggesting that asGM1 positive cells, through the production of soluble helper factors, contribute to the differentiation of alloimmune CTL. In contrast, neither IL 2-containing Con A

supernatant nor irradiated PV-primed helper spleen cells were able to reconstitute the generation of PV-specific CTL from anti-asGM1 and complement treated responder spleen cells. Since IL 2-containing supernatants were able to restore helper function to CY-treated, PV-primed spleen cells (but not anti-asGM1 depleted spleen cells), it seems likely that CTLp (or perhaps both CTLp and T helper precursors) express asGM1.

Since asGM1 appeared to be expressed on CTLp, attempts were made to abrogate the generation of PV-induced NK and CTL activities by in vivo administration of the antibodies. A single injection of anti-asGM1 6 hr before primary or secondary infection with PV spared the virus-specific CTL response, but completely eliminated NK activity, thus confirming earlier results by others (Bukowski et al., 1983). Elimination of NK activity in vivo without any diminution in CTL responsiveness could be interpreted as supporting a distinction between NK cells and CTL. However, it is also possible that the antiserum did not actually eliminate NK cells, but rather modulated their activity, perhaps by interfering with the NK lytic mechanism. Interestingly, generation of in vivo CTL responses could also be prevented if anti-asGM1 antibodies were given at daily intervals after infection with PV. This suggests that the timing of the single antibody injection was inappropriate, or that a higher concentration of anti-asGM1 was required to eliminate the CTL response. The former possibility is favoured because titration of the antibody on NK cells and CTL (taken at 4 and 7 days after primary PV infection, respectively) revealed that they express similar amounts of asGM1.

Previous studies have shown that asGM1 is expressed in high

concentrations on endogenous NK cells compared to alloimmune CTL, and that this difference is large enough that anti-asGM1, together with complement, could be used as an NK-specific antibody (Kasai et al., 1980a; Young et al., 1980). It is not clear why, in the experiments presented here, virus-induced NK and CTL effectors appeared to have similar sensitivities to anti-asGM1 in the presence of complement. One explanation was that the commercially prepared antibody used in these studies is not strictly specific for asGM1 (i.e. contaminating antibodies to a T cell marker distinct from asGM1 could account for the observed activity of this antiserum against NK and CTL effectors). This is unlikely, however, as absorption of anti-asGM 1 with micelles containing purified antigen removed reactivity against both NK cells and CTL. Another possibility was that anti-viral CTL express more of this marker than do alloimmune CTL, but titration studies using PV-specific and allospecific CTL did not reveal any difference in susceptibility to the antiserum plus complement. Finally, it is possible that virus-induced NK cells are relatively more resistant to treatment with anti-asGM1 and complement than are endogenous NK cells. The observation that LCMV-induced NK activity is less sensitive to treatment with anti-asGM1 and complement than endogenous NK activity supports this contention.

The ability of anti-asGM 1 antiserum to eliminate PV-specific CTL activity in vivo (and in vitro in the presence of complement) indicates that asGM 1 is not a strict marker for NK cells in virus-infected mice. This view is supported by the recent studies of Yang et al. (1985), who demonstrated by immunofluorescence that murine spleen cells with LCMV-specific CTL activity are asGM 1

positive, and that virus-specific CTL can be eliminated by treatment with anti-asGM 1 and complement. Shared expression of asGM 1 by virus-induced NK cells and CTL could suggest that a close lineage relationship exists between these two populations of cytotoxic lymphocytes. Alternatively, since asGM 1 has been found on a variety of cell types (including granulocytes, gut epithelial cells, and brain cells), it is possible that NK cells are simply an additional population of asGM 1 positive cells that are not necessarily related to the T lymphocyte lineage.

4.3.2 Generation of memory cell-mediated immune responses in CY-treated, NK-primed mice.

Treatment of mice with CY 18 hr before primary infection with PV delayed the appearance of peak NK activity to day 7, and completely abrogated CTL generation. The ability of CY to suppress virus-induced NK activity could be due to a toxic effect of the drug. Alternatively, CY could act by inhibiting NK cell division, or by delaying the production of IFN after virus infection. Nevertheless, pretreatment with CY allowed the specific priming of the virus-induced NK response in the absence of detectable CTL activity, thus permitting separation of the NK and CTL compartments. It was reasoned that if virus-induced NK represent CTLp, then rechallenge of NK-primed mice should result in the generation of secondary NK and CTL responses. Memory NK activity was detected 1 day after reinfection of NK-primed mice, but this response was accompanied by a markedly diminished CTL response that persisted through 7 days after rechallenge with PV. One interpretation of this result is that NK and CTL activities are mediated by distinct subsets of lymphocytes, and that only the CTL

subset is sensitive to treatment with CY. Alternatively, CY could block the differentiation of memory virus-induced NK cells into CTL. Potential mechanisms that could result in such a block include the establishment of a persistent PV infection, interference with the generation of T cell help, or activation of suppressor cells.

Other investigators have shown that mice treated with CY before LCMV infection become persistently infected and are unable to generate LCMV-specific CTL responses (Gilden et al., 1972). In contrast to these findings, it has been demonstrated that CY treatment delayed the clearance of PV from mice by several days, but did not result in the establishment of persistent infection. Low titers of PV could occasionally be detected in pooled lymph node homogenates several weeks after infection, but it is unlikely that this could account for the long-term block in CTL responsiveness.

The CY-induced block in anti-PV CTL generation could be overcome by co-infecting mice with certain viruses at the same time as reinfection with PV, or by providing IL 2-containing supernatants. This result suggests that CY inhibits the activity of an IL 2-producing T helper cell subset, resulting in long-term CTL unresponsiveness. A number of other investigators have previously shown that induction of in vitro anti-viral CTL activity requires the participation of T helper cells that produce soluble factors such as IL 2 (Ashman and Mullbacher, 1979; Reiss and Burakoff, 1981; Pfizenmaier et al., 1980; Schmid and Rouse, 1983). Furthermore, studies with chimeric mice suggest that H-2I region-restricted helper T cells are necessary for the induction of primary in vivo CTL responses specific for viruses (Zinkernagel et al., 1978; Howes et

al., 1979; Finberg et al, 1979; von Boehmer et al., 1978; Matsunaga and Simpson, 1978). The data presented here provide the first evidence that such a helper cell population is also required for the generation of secondary in vivo CTL responses.

Kinetic studies showed that memory anti-PV CTL activity peaked 4 days after coinfection, and subsided thereafter. Thus, coinfection resulted in the generation of a memory CTL response despite the fact that a primary CTL reponse could not be detected in CY-treated, PV-primed mice. Since these mice did generate NK responses after primary infection with PV, one might speculate that priming the NK compartment is sufficient to prime for a memory CTL response, as long as helper factors (such as IL 2) are supplied.

Non-specific helper factors produced in response to the coinfecting viruses are presumably responsible for restoration of PV-specific CTL activity in CY-pretreated mice. Interestingly, coinfection with other arenaviruses (such as LCMV or TV) was more efficient in providing helper activity than coinfection with VSV, an unrelated rhabdovirus. If PV-specific CTLp are receptive to helper signals for a short period of time after re-exposure to PV, then it might be expected that the timing of factor production is more suitable when another arenavirus is used as the coinfecting virus. Alternatively, arenaviruses may cross-react at the helper T cell level. This would imply that arenavirus-specific T helper cells and CTL have different antigen recognition repertoires, as data presented here (table 3) and elsewhere (Buchmeier et al., 1980) show that CTL elicited by one arenavirus will not lyse a target cell infected with other viruses of the same family (i.e. there is no cross-reactivity at

the CTL level). This situation would be distinct from the influenza system, where the T helper and CTL subsets appear to have similar antigen recognition patterns (Ashman and Mullbacher, 1979; Askonas et al., 1982).

The finding that IL 2-containing supernatants restore anti-PV CTL responses both in vitro and in vivo lends strong support to the argument that CY blocks T helper cell responsiveness. IL 2 appears to be the active factor required in these supernatants, as IL 2 purified to apparent homogeneity, when added to cultures of spleen cells from CY treated mice, will promote the generation of anti-PV CTL. However, this result should not be interpreted as proof that IL 2 is the only factor required for the induction of CTL activity; it is possible that other factors such as TCDF are also required but their production is not affected by CY treatment (Raulet and Bevan, 1982; Wagner et al., 1982; Mannel et al., 1983; Kanagawa 1983; Finke et al., 1983; Garman and Fan; 1983; Hardt et al., 1985). The finding that CY selectively inhibits IL 2 production is consistent with previous reports showing that tumor-specific CTL responses in the draining lymph node of CY-treated mice could be restored in vivo and in vitro by administration of IL 2-containing supernatants or highly purified human IL 2 (Merluzzi et al., 1981a; 1981b; 1983; Hancock and Kilburn, 1982). Similarly, Varkila and Hurme (1983) have shown that CTL responses to minor alloantigens or TNP-modified syngeneic cells are abrogated in CY-treated mice but could be reinstated when IL 2-containing supernatants were provided. It is not clear how CY inhibits the activity of IL 2-producing T helper cells. Treatment with CY, a DNA alkylating agent, could destroy rapidly proliferating T

helper cells. Alternatively, CY could impose a functional block on T helper cells without actually eliminating them. This could include the induction of a suppressor cell population that specifically blocks the generation of PV-specific T helper cell responses. Presumably such a suppressor mechanism is circumvented if IL 2 is supplied.

In addition to affecting T helper cells, CY treatment may affect the generation of a CTL response by decreasing the number of CTLp. Recently, other investigators have shown by limiting dilution analysis that tumor- and influenza virus-specific CTLp are markedly reduced after CY treatment (Hancock and Kilburn, 1982; Merluzzi et al., 1984b). The number of memory anti-PV CTLp also appears to be reduced when CY is administered to mice before primary PV infection. Indeed, by limiting dilution analysis the frequency of CTLp capable of responding to PV drops by approximatelty 50-fold after pretreatment with CY. It has been argued that CY reduces the number of antigen-specific CTLp, but that survivors are expanded in the presence of added IL 2 to give a detectable CTL response (Hancock and Kilburn, 1982; Merluzzi et al., 1984b). This interpretation appears to be at odds with the hypothesis that PV-induced NK activity is mediated by CTLp (i.e. if CTLp frequencies are markedly reduced in CY-treated, PV-primed mice, then rechallenge with homologous virus would not be expected to elicit normal levels of memory NK activity). Thus, it seems possible that the frequency of CTLp is not actually reduced, but that helper cells or soluble factors not present under limiting dilution conditions are necessary for differentiation of NK active CTLp to the mature CTL stage. This activity is presumably distinct from IL 2 (which was present in excess in limiting dilution cultures),

and could be mediated by an antigen-specific helper factor or possibly a T cell differentiation factor. CTLp from mice not treated with CY probably would not require this additional helper activity under limiting dilution conditions, since the helper cells producing this factor would function normally in the absence of CY. If this hypothesis is correct, then one might predict that an excess of irradiated "helper" spleen cells from CY-treated or untreated PV-primed mice, when added to limiting dilution cultures of spleen cells from CY pretreated mice, would result in the generation of a CTLp frequency that is close to that of PV-primed mice that had not been treated with CY.

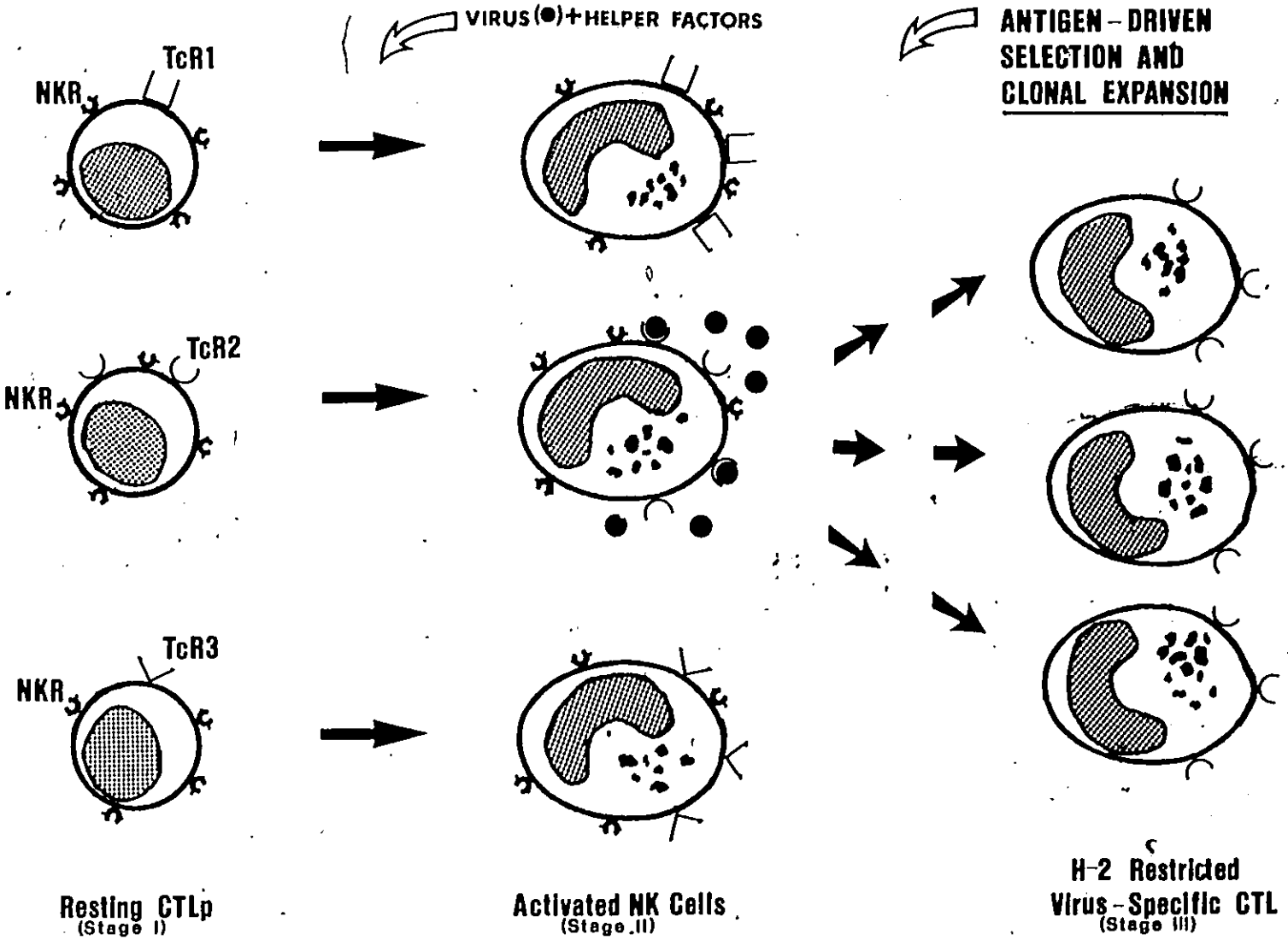
4.4 Summary and Conclusions

Relationship between NK and CTL effectors: proposed model. The data presented in this study suggest that there is a relationship between virus-induced NK activity and CTL. To summarize, the temporal kinetic relationship between NK and CTL generation is maintained after both primary and secondary infection with PV, even though the memory responses developed more rapidly. In addition, by pretreating mice with CY prior to primary infection with PV, the NK compartment was primed without the generation of a detectable CTL response; rechallenge of these mice with homologous virus resulted in the generation of memory NK activity and a CTL response, provided growth factor (in the form of IL 2) was supplied. Furthermore, the pan T cell marker Thy-1 is acquired by virus-induced NK cells as the primary or secondary PV infections progress.

Based on these studies, it is proposed that virus-induced NK activity (as detected against YAC-1 target cells) is mediated by

activated CTLp. According to this model, each CTLp can exhibit distinct antigen-recognition receptors. These would include a TcR that is able to recognize antigen in the context of self-MHC, and a second receptor, or set of receptors, that allow recognition and lysis of NK-sensitive target cells. The antigen specificity of the TcR would be genetically determined and clonally distributed. In contrast, the NK receptor(s) would be present on most or all CTLp regardless of the antigen-specificity imposed by the TcR (i.e. receptors for NK sensitive targets would be polyclonally distributed on CTLp). Whether a given CTLp kills specific targets or NK sensitive targets would depend on its stage of differentiation and/or activation. For instance, infection of mice with a virus (such as PV) would result in a polyclonal activation of CTLp, with a corresponding increase in NK activity against YAC-1 cells (figure 10, stage 2 cells). This activation step could be mediated solely by soluble factors (such as IFN) produced in response to the virus. Furthermore, given the broad target cell spectrum that virus-induced NK cells exhibit, it seems possible that polyclonally activated CTLp (i.e. stage 2 cells) can lyse targets using either NK-specific or antigen-specific receptors. However, only those CTLp clones expressing the relevant TcR would be selected for further differentiation and clonal expansion to become virus-specific, H-2 restricted CTL (i.e. stage 3 cells). The lytic activity of clones with inappropriate TcR would rapidly return to background levels. In this model, endogenous NK activity can be accounted for, at least in part, by low levels of IFN (and other NK-augmenting factors) that are produced in response to chronic stimulation with environmental

Figure 10. Virus-induced NK activity is mediated by activated CTLp: Proposed Model. Legend: NKR (ç)- polyclonally distributed receptor(s) for NK-sensitive target cells such as YAC-1. TcR (∪/⌊⌋/⌊_)- clonally distributed, H-2 restricted T cell receptor for antigen. See discussion section for details.


VIRUS (●) + HELPER FACTORS
ANTIGEN – DRIVEN SELECTION AND CLONAL EXPANSION
TcR1
NKR
TcR2
NKR
TcR3
NKR
Resting CTLp
(Stage I)
Activated NK Cells
(Stage II)
H-2 Restricted
Virus-Specific CTL
(Stage III)

antigens. However, it is also possible that T cells at various other stages of differentiation could also contribute to endogenous NK activity (discussed below).

The availability of techniques for cloning NK and CTL should permit direct examination of this model. It should be possible to grow clones with NK (i.e. anti-YAC-1) activity that, when exposed to PV, become H-2 restricted, and virus-specific in their killing pattern. Indeed, preliminary evidence suggests that cells with NK activity taken from CY treated, PV-primed mice can be grown in long-term culture in the presence of IL 2 (KLR and CMW, unpublished observation). These cells appear to lose NK activity and become H-2 restricted, PV-specific CTL when exposed to virus infected stimulator cells (Rosenthal et al., 1985), although this work must be repeated using cloned cell populations. When such a cell system is developed, it should be possible to detect, at a single cell level, clones that are able to lyse both YAC-1 and syngeneic, PV-infected target cells. One further prediction of this model is that NK-active clones have TcR genes that are rearranged and expressed. This should be testable using cDNA probes for the TcR alpha, beta, and, gamma genes, and with antibodies to their respective proteins.

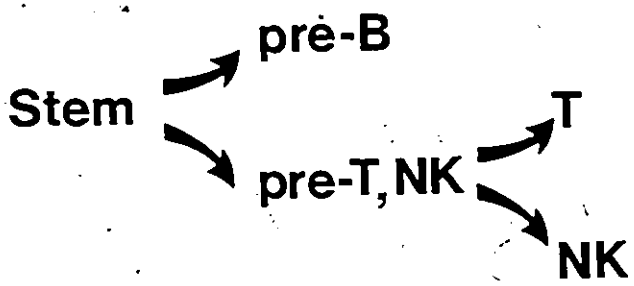
It is important to point out that T cells at various other stages of differentiation could also mediate NK activity. For instance, NK and T cells could share a common precursor that differs from the B cell precursor, but T and NK cells develop independently (figure 11A; Pollack et al., 1979; Habu and Okumura, 1982). Alternatively, the observation that cloned CTL lines lose antigen specificity in culture, and acquire NK cell-like morphology and

surface markers, suggests that NK activity is mediated by terminally differentiated CTL (figure 11B; Brooks, 1983; Brooks et al., 1983b). NK activity could also be mediated by immature T cells (such as pre-thymocytes or thymocytes) that are not yet immunocompetent (figure 11C). This view is supported by the observation that some cloned NK cell lines resemble immature thymocytes in that TcR beta chain genes but not TcR alpha chain genes are rearranged (Ritz et al., 1985). Further, some NK cells express immature T cell differentiation antigens, and nude mice (which lack a thymus) have high levels of NK activity (reviewed by Ortaldo and Herberman, 1984). Another possibility is that NK cells can undergo further maturation extrathymically to become H-2 restricted CTL (figure 11D). The observation that antigen-specific CTL can be generated from spleen cells of athymic nude mice when cultured with appropriate growth factors supports this hypothesis (Gillis et al., 1979; Skinner and Marbrook, 1983; Maryanski et al., 1981). Thus; there could be two pools of CTLp; one that is derived from cells that have undergone thymus-dependent maturation, and another that has undergone thymus-independent maturation (Grossman and Herberman, 1982). It is important to point out that these various options are not mutually exclusive, and that T cells at various stages of differentiation and/or activation could express NK activity.

Reconstitution of virus-specific cytotoxic cell responses in CY-treated, PV-primed mice: therapeutic implications. It has already been mentioned that a variety of mechanisms could account for the lack of virus-specific CTL responses in mice persistently infected with LCMV, including clonal deletion of virus-specific CTLp (Dunlop and

Figure 11. Possible lineage relationships between NK cells and T lymphocytes. See discussion section for details.

A.


pre-B
Stem
T
pre-T, NK
NK

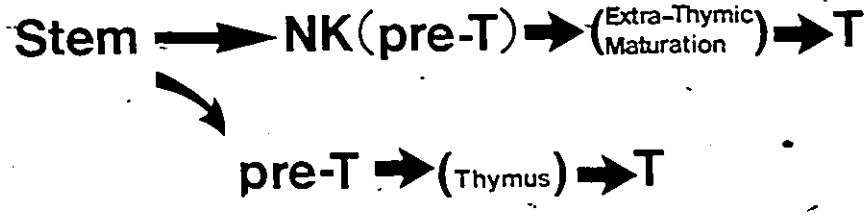
B.


CTLp
CTL
NK
?

C.


Stem
NK(pre-T)
T

D.


Stem
NK(pre-T)
(Extra-Thymic Maturation)
T
pre-T
(Thymus)
T

Blanden, 1977; Cihak and Lehmann-Grube, 1978) and generation of T suppressor cells (Zinkernagel and Doherty, 1974b). It is also possible that persistence results from inadequate T helper cell function. In light of the finding that coinfection or IL 2 plus virus can bypass the requirement for T helper cells in vivo, it would be useful to determine if a similar approach could be used to cure persistent LCMV infection. If so, this method could also be considered as a specific treatment for persistent viral infections of humans where the basic underlying defect contributing to persistence may also be impaired T helper cell activity. Thus, persistent infections with hepatitis virus, measles virus (SSPE), congenitally-acquired rubella virus or the AIDS-associated retrovirus might be treatable with virus vaccine plus IL 2.

CY has been used effectively in the treatment of certain human cancers (Gershwin et al., 1974; Fischer, 1982). However, its therapeutic effect is compromised by its ability to cause immunosuppression, which results in increased susceptibility to infectious disease. These studies indicate that the ability to generate anti-viral CTL responses may be restored in CY-treated mice by administration of IL 2. Thus, these studies support the contention that IL 2 therapy may be useful in combination with CY to lessen the negative impact of the drug on the cellular immune system of cancer patients (Merluzzi et al., 1984b).

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