GENETIC AND VIRAL SPECIFICITY OF THE CELL-MEDIATED IMMUNE RESPONSE TO PICHINDE VIRUS IN MICE

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By
Cathy Robin Steiner, B.Sc.

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AUTHOR: Cathy Robin Steiner
B.Sc. (University of Toronto)

SUPERVISOR: Dr. K. L. Rosenthal

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ABSTRACT

This study was undertaken to examine the genetic and viral target specificity of the cell-mediated immune response to Pichinde virus (PV) in mice, to ultimately facilitate the cloning of anti-PV cytotoxic T lymphocytes (CTL). Cloned populations provide a system for the study of the generation, function, and mechanism of action of CTL in viral infections.

CTL are often genetically restricted to either the K or D end of H-2 Class I. Cytotoxicity assays performed using primary immunized murine splenocytes showed predominant restriction to the K locus in H-2^b C57Bl/6 mice. In limiting dilution assays, a significant population of anti-PV CTL (approximately one-half) were regulated by H-$2K^b$.

A panel of temperature sensitive (ts) mutants of wild type (wt) PV was used to study the viral target specificity of anti-PV CTL. These mutants were grouped according to surface expression and internal production of viral molecules as detected by indirect immunofluorescence and polyacrylamide gel electrophoresis respectively. Cytotoxicity assays with target cells infected with these mutants provided

equivocal results regarding whether the major target antigen was an internally or externally expressed viral protein. The results suggest that surface expression of viral proteins may be related to target specificity in the anti-PV cytotoxic response.

One temperature sensitive mutant, ts 488, provided a superior target for H-2^b effectors than wt PV when used to infect 5R (H-$2K^b$ compatible) target cells. Limiting dilution studies indicated that anti-ts 488 CTL were predominantly regulated by H-$2K^b$: approximately three-quarters of the CTL precursors were restricted to that end. Furthermore, limiting dilution assays indicated that ts 488 and wt PV were not fully cross reactive in generating lytic effectors. As a result, the mutant might not facilitate the generation of cloned lines of anti-wt PV CTL.

The mutant and its augmented regulation by H-$2K^b$ may provide a novel system for the examination of the association and recognition of viral plus "self" antigens at the level of antigen presentation and target identification.

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"The goal of yesterday will be
the starting point of tomorrow"

- Carlyle

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

APC	antigen presenting cells
CMI	cell-mediated immunity
CTL	cytotoxic T lymphocytes
CTLp	cytotoxic T lymphocyte precursors
GPC	virus-encoded glycoprotein
IFN	interferon
IL-1	interleukin-1; lymphocyte activating factor
IL-2	interleukin-2; T-cell growth factor
i.v.	intravenous
LAK	lymphokine activated killer cells
LCMV	lymphocytic choriomeningitis virus
LDA	limiting dilution assay
MHC	major histocompatibility complex
NC	natural cytotoxic cells
NK	natural killer cells
NP	virus-encoded nucleoprotein
PEC	peritoneal exudate cells
PV	Pichinde virus
TcR	T-cell receptor for antigen + MHC
Th	helper T lymphocytes
ts	temperature sensitive mutant virus strain
VSV	vesicular stomatitis virus

A./ INTRODUCTION

A/1. PICHINDE VIRUS

Pichinde virus (PV) was first isolated from the rodent Oryzomys albigularis, native to the Pichinde Valley of Columbia (Trapido and Sanmartin, 1970). This virus was added to the group of Arenaviridae based on similar virology, epidemiology, and pathogenesis.

A/1.1. Virology

A/1.1.1. Morphology

The Arenavirus family derives its name from arenosus (L.,sandy) to reflect the numerous granules observed in ultrathin sections of virus preparations (Rowe et al, 1970). Typically, the spherical, oval, or pleomorphic virions (Murphy and Whitfield, 1975) may range in diameter from 50 to 300 nm, but are generally 110 to 130 nm wide (Rawls and Buchmeier, 1975; Rowe et al, 1970). During budding virions are enveloped by the bilayer plasma membrane of host cells (Murphy and Whitfield, 1975) which contains virus-encoded glycoproteins (Rowe et al, 1970). The nucleic acid is single stranded RNA, based on its sensitivity to ribonuclease and alkali, buoyant density in cesium sulphate, and base composition (Carter et al, 1973). In place of a discernible virion core, electron dense granules of 20 to 25 nm diameter are observed (Rowe et

al, 1970; Rawls and Buchmeier, 1975; Vezza et al, 1977). They are relatively stable at physiologic temperature and pH, and can tolerate multiple cycles of freezing and thawing (Mifune et al, 1971).

A/1.1.2. Nucleic Acid

Six species of RNA have been described for PV. They have sedimentation coefficients of 31S, 28S, 22S, 18S, 15S, and 4 to 6S (Dutko et al, 1976; Vezza et al, 1977). The observed sensitivity of 28S and 18S RNA to actinomycin D suggests that they are of host ribosomal RNA origin. Additionally, these species are methylated, characteristic of cellular ribosomal and transfer RNA (Carter et al, 1973). In point of fact, density gradient centrifugation followed by polyacrylamide gel electrophoresis illustrated that 28S and 18S RNA's could be extracted from the fractions containing 80S monosomes of host BHK-21 cells. Furthermore, 28S and 18S RNA's were apparently derived from host 60S and 40S ribosomal subunits respectively (Farber and Rawls, 1974). Electron micrographs indicate that viral granules morphologically resemble ribosomes (Murphy and Whitfield, 1975). The 4 to 6S RNA is methylated as well (Carter et al, 1973), but it is not actinomycin D sensitive. This suggests that it may be ribosome-associated material (Pedersen, 1979). In contrast, it has been suggested that the granules are core material

altered to look like ribosomes (Lehmann-Grube, 1984). Studies of a hamster embryonic lung cell line displaying a temperature-sensitive defect in the 60S ribosomal subunit indicate that the ribosomes incorporated by PV virions are not required for replication, but may alternatively be cellular contaminants incorporated during budding (Leung and Rawls, 1977).

The 31S and 22S RNA species (Pedersen, 1979) and the 15S RNA species described in some reports (Vezza et al, 1977) are encoded by the viral genome. Both the 31S and 22S RNA's lack methylated nucleotides and a 3' polyadenosine cap, rendering them noninfectious (Vezza et al, 1977). The 31S large (L) segment of the genome is approximately 2.63×10^6 to 2.83×10^6 molecular weight, as estimated by denaturing polyacrylamide gel electrophoresis. Similarly, the 22S small (S) segment has an estimated molecular weight of 1.26×10^6 to 1.31×10^6 daltons (Ramsingh et al, 1980).

A/1.1.3. Proteins

At least four polypeptides have been identified by polyacrylamide gel electrophoresis under denaturing conditions (reviewed in Howard and Simpson, 1980), only three of which are primary gene products (Harnish et al, 1981).

One polypeptide, the nucleoprotein (NP), is nonglycosylated (Harnish et al, 1981), and has a

molecular weight of approximately 72,000 daltons (Ramos et al, 1972; Rawls and Buchmeier, 1975).

Another large but minor polypeptide of approximately 72,000 (Ramos et al, 1972) to 75,000 (Howard and Simpson, 1980) molecular weight is a glycoprotein, as observed by the incorporation of glucosamine (Ramos et al, 1972). It has been suggested that this polypeptide is the precursor of the two surface glycoproteins (GPC) via proteolytic cleavage. GP1, of 64,000 to 72,000 molecular weight, as well as GP2 of 34,000 to 38,000 daltons, are the glycoproteins found on the virion envelope (Harnish et al, 1981).

Results of tryptic mapping of reassortant strains of PV indicate that the primary transcripts of NP and GPC are encoded by the S segment (Harnish et al, 1983). Accordingly, use of high pressure ion exchange column chromatography to resolve tryptic peptides led to the same conclusion (Vezza et al, 1980). This part of the genome is described as ambisense because NP is encoded in a sequence complementary to the virus, corresponding to the 3' half of the S RNA. The second S RNA gene product, GPC, is encoded by a non-overlapping virus-sense subgenomic mRNA. It has been suggested that a hairpin region between the two acts to stop transcription (Auperin et al, 1984).

The virus contains no DNA polymerase, but an RNA

dependent RNA polymerase activity, similar to that of influenza (Carter et al, 1974). This transcriptase transcribes a heterogeneous range of RNA species complementary to virion RNA and transcriptase product RNA. It has been suggested that the L RNA may code for a polymerase component (Auperin et al, 1984).

A/1.2. Epidemiology

Arenaviruses are loosely divided into two groups based on degree of homology, shared complement-fixing antigens, and origin. Lymphocytic choriomeningitis virus (LCMV) and Lassa fever virus exhibit a great degree of homology and are considered Old World Arenaviruses. Correspondingly, the Tacaribe group, including Tacaribe, Junin, Machupo, Amapari, Parana, Tamiami, Latino, and PV exhibit morphological similarities and are considered New World Arenaviruses, owing to their origin in the Americas (Lehmann-Grube, 1984).

Each Arenavirus is carried by a natural rodent host (Rowe et al, 1970). LCMV is associated with a single murid species, and has become quite well distributed globally. Conversely, Lassa fever virus is generally localized in Africa, with its reservoir host being the common African rodent mastomys. Members of the Tacaribe group of Arenaviruses are common in South America, and

are carried by a variety of cricetid rodents (Arata and Gratz, 1975). PV is naturally found in Oryzomys albigularis (Lehmann-Grube, 1984). Horizontal and vertical intraspecific virus transmission appears to be the fundamental mechanism for natural maintenance of these infections (Johnson et al, 1973).

A/1.3. Pathogenesis

A/1.3.1. Virus-Cell Interaction

Typically, Arenaviruses are lymphotropic, infecting reticular cells and megakaryocytes in lymphoid organs shortly after infection (Murphy and Whitfield, 1975), as well as hepatic Kupffer cells. The extent of infection of these cell types may determine the outcome of the disease (Murphy et al, 1977).

Studies of PV replication in BHK-21 cells have shown that an intact host nucleus is required for at least eight hours post infection (Banerjee et al, 1975,6). Additionally, a rapid increase in viral polypeptide synthesis is observed, peaking one to three days post-infection. Within ten days after exposure, less than one per cent of cells release infectious virus, with ninety per cent containing viral nucleoprotein or a related polypeptide. An increase in the infecting dose will accelerate this process, suggesting regulation either by defective interfering

particles, or by expression of some late gene products (Dimock et al, 1982).

A/1.3.2. Virus-Host Interaction

Members of the Arenavirus family typically cause a chronic carrier state in their natural rodent hosts (Rowe et al, 1970). In the state of prolonged viraemia, it is difficult to demonstrate neutralizing antibodies in immune fluids (Trapido and Sanmartin, 1971).

As a human pathogen, Arenaviruses may cause lethal haemorrhagic fevers (Lassa, Junin, etc.), aseptic meningitis (LCMV) or no symptoms (PV). Experimental transmission of PV by infection of a neonatal animal may lead to cytopathology and death. Conversely, infection of an adult animal induces an immune response that may eventually lead to viral clearance and recovery. (Lehmann-Grube, 1984).

The major complement-fixing antigen of PV appears to be a cleavage product of viral structural proteins of approximately 20,00 to 30,000 molecular weight. These complement fixing antigens may well be internal, resulting in a deficient neutralizing antibody response (Buchmeier et al, 1977). Observations of other systems such as LCMV indicate that neutralizing antibodies are induced predominantly by surface glycoproteins (Lehmann-Grube, 1984).

Studies of susceptibility of hamsters to PV

infection have shown that the nature of the host immune response to this virus may be determined by genetic factors. PV induces an asymptomatic infection in the LVG strain, and a lethal infection in the MHA strain (Buchmeier and Rawls, 1977). Treatment with cyclophosphamide causes inhibition of cell division and either a loss of LVG resistance, or a further increase in MHA susceptibility. Taken together, this data suggests that the outcome of a hamster PV infection is determined not by cytopathology, but by an effective cellular immune response which is genetically determined. (Murphy et al, 1977).

A/2. THE CELLULAR IMMUNE RESPONSE

Cell mediated immunity (CMI) consists of specific lysis of targets expressing foreign antigen as well as self molecules by cytotoxic T lymphocytes (CTL), in addition to less specific lysis by natural killer (NK) cells, natural cytotoxic (NC) cells, and lymphokine activated killer (LAK) cells. Whereas antibodies are stimulated by soluble native proteins, CTL are stimulated by determinants preserved after protein denaturation that are expressed at the cell surface with self Class I H-2 molecules in the mouse or HLA-A,B, and C in the human (Germain, 1986). Conversely NK, NC, and LAK responses lack this specificity for antigen and self

molecules.

The response of CTL to antigen may be described in three stages: antigen presentation, growth and differentiation, and finally the effector stage.

A/2.1. Cytotoxic T Lymphocytes (CTL)

A/2.1.1. Antigen Presentation

Cytotoxic T lymphocytes (CTL) are activated by antigen, interleukin 1, and the cooperation of interleukin 2 - secreting helper T lymphocytes (Th). Th are stimulated following recognition of self class II restricting elements (Ia in the mouse, HLA-D in the human) coupled with antigenic determinants preserved after protein denaturation (Germain, 1986). This immunogenic stimulus occurs when antigen is expressed on cells of lymphohemopoietic origin (Zinkernagel et al, 1980). Class II MHC molecules are expressed predominantly on cells of the monocyte-macrophage lineage, and dendritic cells, of which either can function as an antigen presenting cell (APC) (Katz et al, 1986). In fact, any cell type may process antigen for presentation by a class II - bearing cell, with various cell types interacting to initiate a cellular response (Roska and Lipsky, 1985).

Antigen processing involves denaturation of immunizing proteins. It appears to reflect a

fundamental set of intracellular activities occurring in virtually all cells. Antigen is internalized either by pinocytosis, or by receptor mediated endocytosis. The ubiquitous endosomal recycling pathway may have a predominant role in processing; whereby, the endosomes acidify thus denaturing and fragmenting the original protein (Germain, 1986). There does, however, appear to be more than one processing pathway to form a particular antigenic determinant (Kim et al, 1985). Once the endogenous antigens are denatured, most are transported to lysosomes and are completely degraded, while some are transported to the plasma membrane and stimulate CMI. These antigenic determinants may be transported either alone, or already associated with antigens of the major histocompatibility complex (MHC). In either case, processing is presumed to increase the lipophilicity of the antigen, thus stabilizing its interaction with the plasma membrane and maximizing the effective local concentration of antigen in the environment of MHC (Germain, 1986).

A/2.1.2. Growth and Differentiation

Macrophages activated by antigen produce interleukin 1 (IL 1), formerly known as lymphocyte activating factor. This signal, combined with antigen presentation, activates a population of Th (Kouttab et al, 1984). Th cells are generally characterized by

surface L3T4 or Lyt1 in the mouse, and CD4, T4, or Leu3 in the human (Townsend, 1985). In turn, these Th secrete interleukin 2 (IL 2), formerly known as T-cell growth factor. This lymphokine continues to stimulate Th cells in an autocrine regulatory pathway (Cohen, 1986). Additionally, this lymphokine promotes the proliferation of CTL (Vohr and Hunig, 1985; Wagner et al, 1982).

This prerequisite factor for growth of T cells was first determined in vitro using conditioned supernatant from mitogen-stimulated spleen cells (Morgan et al, 1976; Gillis and Smith, 1977). Since the factor has been identified as IL 2, its role has been more precisely characterized.

IL 2 acts in an autocrine fashion to upregulate expression of IL 2 receptors on Th cells (Malek and Ashwell, 1985; Reske-Kunz et al, 1986), as well as on CTL (Lowenthal et al, 1985). The interaction between IL 2 and its cell surface receptor is required for DNA synthesis and mitosis, since antibodies to the receptor inhibit IL 2 binding and cell proliferation. In addition, the concentrations of IL 2 that bind to receptors coincide exactly with those that promote T cell proliferation (Cantrell and Smith, 1984). IL 2 mediates completion of the T cell mitotic cycle from the G1 to the S phase (Reske-Kunz et al, 1986). The

mechanism of this response is similar to a number of receptor-ligand interactions. Following the binding of IL 2 to the cell surface receptor, the membrane mobility of the cell increases, concommitant with the breakdown of phosphatidylinositol bisphosphate into two second messengers: inositol triphosphate and diacylglycerol. Subsequently, calcium is released from intracellular endoplasmic reticulum into the cytoplasm via a signal from inositol triphosphate. Finally, there is an influx of calcium from the external medium into the cytoplasm. Diacylglycerol, which stimulates protein phosphorylation, may mediate the opening of membrane calcium channels, and the ultimate physiologic responses (Utsunomiya et al, 1986).

The role of IL 2 in CTL proliferation is certain. There is, however, controversy over whether it is a sufficient signal to induce differentiation of resting precursors to active CTL. In a system employing lectin-derived CTL, IL 2 was sufficient for both growth and differentiation (Vohr and Hunig, 1985). Other groups report the need for a factor found in supernatants from mitogen-stimulated spleen cells but distinct from IL 2: cytotoxic T-cell differentiation factor (Wagner et al, 1982), and CTL differentiation factor (Raulet and Bevan, 1982). Yet other groups describe the role of two factors besides IL 2, also derived from the supernatant

of stimulated spleen cells: T-cell cytotoxicity-inducing factors 1 and 2 acting in the first and last 48 hours of culture respectively (Mannel et al, 1983), as well as IL 2 receptor-inducing factor and cytotoxic T-cell differentiation factor (Hardt et al, 1985). In addition, a role for interferon (IFN) alpha and gamma in CTL differentiation has been described (Chen et al, 1986).

Certain activation antigens have been associated with differentiated CTL. Expression of surface oligosaccharides appears to be functional, as it can be regulated by IL 2. Additionally, the molecules are specific for a given cell type. Of these glycoproteins, T200 has been identified by a monoclonal antibody. It is CTL specific and inhibition studies implicate it in cytotoxicity (Lefrancois and Bevan, 1985). Other such determinants associated with the T200 glycoprotein and secreted glycoproteins have also been reported as markers of differentiation (Lefrancois et al, 1985).

A/2.1.3. The Effector Stage

Binding the target. Binding of the specific T-cell receptor (TcR) to the target is a prerequisite for initiating the lytic function, since monoclonal antibodies which bind the receptor can inhibit cytolysis (Eisen et al, 1985). The TcR (reviewed in Samelson et al, 1985; Leiden et al, 1986) is a disulfide-linked

heterodimeric glycoprotein consisting of an acidic alpha chain and a basic beta chain, each of approximately 40,000 to 50,000 daltons in molecular weight. The α and β chains belong to the immunoglobulin supergene family, thus exhibiting a variable and a constant region domain linked to a hinge-like region, followed by a transmembrane region and a short cytoplasmic tail. This structure is analogous in both human and murine T cells. In the human, the TcR is noncovalently associated with three other proteins of the T3 complex (Samelson et al, 1985). Similarly, four polypeptides are noncovalently associated with the murine receptor: a glycoprotein γ chain of 25,000 daltons, another glycoprotein chain δ of 21,000 daltons, an ε chain of about 22,000 daltons, and a ζ chain of 16,000 daltons which exists as a disulfide-linked 32 kD molecule,probably a homodimer. The δ chain appears to be phosphorylated in response to T-cell activation. The γ chain genes are the first to be transcribed in T-cell ontogeny, and their level of transcription is reduced upon α gene activation (Steinmetz and Dembic, 1986).

The Vβ chain is encoded by multiple variable (V), diversity (D), and junctional (J) gene segments that rearrange during differentiation to form a contiguous message with one of two Cβ genes. At least 30 possible combinations have been predicted. V_α genes

rearrange only V and J segments along with a single C_α segment, yielding at least 100 possible rearrangements (Goverman et al, 1986). Diversity is thus generated by the apparently random combination of segments, in addition to the accuracy of their joining, and somatic mutation (Steinmetz and Dembic, 1986).

The manner in which the α and β chains and their associated proteins function in specific antigen recognition is currently unclear. Expression of both the α and β chain genes has been observed to be necessary and sufficient for CTL specificity (Dembic et al, 1986). However, interferonγ -activated CTL did not exhibit changes in antigen specificity despite new rearrangements of the α chain genes (Chen et al, 1986). Antigen specificity has been observed to change with new rearrangements of β chain genes and expression of altered β chain mRNA (Epplen et al, 1986). As for the role of the gamma chain, an abundance of transcripts are observed in immature thymocytes (Haars et al, 1986), and CTL (Rupp et al, 1986). It was postulated that theγ chain might confer the class I specificity characteristic of CTL; however, in cloned CTL lines, the γcDNA is always out of phase and non-functional (Rupp et al, 1986). Perhaps this gene is functional when expressed in immature progenitors (Reilly et al, 1986).

Whether the TcR complex binds MHC and antigen as

modified self or as two discrete entities remains equivocal. Possibly, other surface molecules may also be involved in binding. In the human system, antibodies to the CD4 or CD8 surface antigen can inhibit cytotoxicity of CD4+ and CD8+ cells respectively. This inhibition can be overcome by high affinity binding to the receptor-T3 complex. Perhaps the negative feedback of CD4 and CD8 offers a control mechanism, preventing lysis of targets which bind them with greater affinity than they bind the receptor complex (Fleischer et al, 1986). Alternatively, it has been suggested that L3T4 and Lyt2 in the mouse, like CD4 and CD8 in the human, may recognize non-polymorphic structures of cellular MHC, thus directing the α / β receptor to antigen presented by that MHC molecule. In this way, accessory molecules may determine MHC class specificity and effector function (Goverman et al, 1986).

Target Cell Lysis. CTL not bound to target cells are morphologically polar. They are motile by extending pseudopods from the broad leading edge which contains the nucleus. Numerous granules reside in the tapered tail. Within two minutes after initial contact is made between the appropriate target cell and the leading edge of the CTL, morphologic changes begin to occur in the latter. The CTL rounds up, and the cytoplasmic granules replace the nucleus in the zone of contact and fuse with

the membrane in the vicinity of the contact area (Yanelli et al, 1986). Delivery of the lethal hit must involve CTL exocytosis because of the dependence on chloride flux (Gray and Russell, 1986). Indeed, the contents of the cytoplasmic granules are able to lyse tumour targets without any specificity (Marx, 1986).

Perforin or pore-forming protein (PFP) has been isolated from CTL granules. Like complement, it appears to mediate target cell lysis by the production of circular lesions with an internal diameter of 160 A (Young and Cohn, 1986). It has been identified as 66kD under non-reducing conditions and 70 to 75 kD under reducing condition (Young et al, 1986a). Assembly of the membrane lesion is dependent on the presence of calcium ions to allow polymerization of the monomer into a functional channel in the membrane (Young and Cohn, 1986). These large aqueous pores are stable, nonselective for solutes, and insensitive to changes in membrane potential (Young et al, 1986b) thus allowing the leakage of water, salts, nucleotides, and proteins across the plasma membrane of the target cell (Young and Cohn, 1986).

In addition to PFP, serine proteases have been identified in the cytoplasmic granules of CTL. By subtractive hybridization, three mRNA transcripts were found almost uniquely in activated CTL, with little or

none in non-cytotoxic lymphoid cells. Serine esterase activity has been associated with the transcripts activated in CTL (Brunet et al, 1986). The serine protease family includes catalytic proteins which convert inactive precursor proteins to active forms by limited proteolysis, as in complement activation and blood coagulation (Neurath and Walsh, 1976). Serine protease activity is associated with the release of calcium from intracellular stores and calcium influx from the external medium into CTL which mediates the subsequent physiologic response (Utsunomiya and Nakanishi, 1986). Proteases could offer greater amplification, specificity, and control in a cascade of lytic molecules, just as the complement enzymes do (Lobe et al, 1986).

One serine esterase of 34 to 36 kD exhibits trypsin-like activity. It is more commonly found as a 60 to 66 kD disulfide-linked homodimer (Young et al, 1986). Another molecule of approximately 28 kD has also been associated with trypsin-like activity. It is 300 times more abundant in cytotoxic T-cells than in non-cytotoxic T-cells, with expression in thymocytes increasing three to four days after stimulation (Pasternack and Eisen, 1985). This enzyme exhibits 25 to 35% homology with members of the serine protease family, and much greater conservation of the active

site, suggesting its involvement in a lytic cascade (Gershenfeld and Weissman, 1986).

CTL granules may mediate target cell lysis by releasing toxic lymphokines. One such cytokine termed lymphotoxin, is not antigen specific. In contrast with polyperforins, it does not require calcium ions to effect cytolysis. It appears to destroy the integrity of the target cell nuclear membrane, ultimately causing DNA release in fragments of approximately two hundred base pairs. This activity is observed in cell-free supernatants, but the presence of cells accelerates cytolysis by approximately 24 hours. Possibly, lymphotoxin exerts its effects inside the cell by entering through the perforin-induced channels (Schmid et al, 1986).

Lymphotoxin is found in heavy (α) and light (β) molecular weight forms. It is antigenically cross reactive with tumor necrosis factor (TNF) (Marx, 1986). In human CTL, an additional cytotoxin has been found which does not cross react with lymphotoxin or TNF (Green et al, 1985; Green et al, 1986).

Since the mediators of cytolysis are, themselves, not antigen specific, the way in which the T-cell imparts specificity remains in question. Binding of the TcR to appropriate antigen and MHC molecules signals the initiation of lysis. But once triggered by the specific

target, a CTL can lyse any other cell that binds to it (Fleischer, 1986; Lanzavecchia, 1986). This implicates the TcR in initiating the lytic machinery, but not in specific delivery of the lethal hit.

Possibly, the contact zone allows for a localized release of cytotoxic substances into a tightly sealed and diffusion-limited microenvironment (Young and Cohn, 1986). This hypothesis is supported by the reorientation of the granules, Golgi apparatus and microtubular organizing complex of the CTL secretory system upon contact with an appropriate target. However, it is curious that CTL apparently lyse only one target at a time, even though more may be bound simultaneously. CTL's recycle by reorienting the lytic machinery, subsequently destroying more targets (Marx, 1986). It is also curious that the CTL is not vulnerable to the released cytotoxic mediators.

An accelerated CTL response of increasing affinity is observed upon subsequent restimulations with homologous antigen <u>in vivo</u> and <u>in vitro</u>. This immunological memory is observed in viral infections exemplified by influenza (Askonas et al, 1982) and LCMV (Dunlop and Blanden, 1976).

A/2.2. <u>Nonspecific Killer Cells</u>

By analogy with the complement system, it has been

suggested that CTL represent the classical pathway, and non-specific killers represent the alternative pathway of CMI activation (Gershenfeld and Weissman, 1986). These non-specific killers include natural killer (NK) cells, lymphokine activated killer (LAK) cells, and natural cytotoxic (NC) cells. Since all these cells are cytotoxic, it has been suggested that they derive from the same lineage, but exhibit variable specificity at different stages of differentiation.

NK cells are a heterogeneous population of lymphoid cells that spontaneously lyse certain tumor cells and virally infected cells through a mechanism unrestricted by MHC molecules (Phillips and Lanier, 1985). In vitro, murine NK activity is measured against YAC-1 lymphoma cells (Hackett et al, 1986). NK cells share surface markers with myelomonocytic as well as T cells (Trinchieri and Perussia, 1984). Murine NK display asialo GM1 and alloantigens of Ly5, QA5, NK1 and NK2, as well as variable levels of Thy1 on their cell surface (Ortaldo et al, 1986).

Young mice generally lack NK activity until they are approximately three weeks old (Merluzzi et al, 1986). Since functional NK cells are found in athymic nude mice, it has been suggested that they may represent CTL precursors which don't mature in the absence of a thymus (Kaplan and Wasserman, 1985). Additionally, like

CTL, NK activity may be amplified by IL 2 (Henney et al, 1981; Kabelitz et al, 1985) and interferon (Djeu et al, 1979). Studies on the majority of cloned NK lines reveal no functional β chain of the TcR, only low levels of a truncated 1 kb mRNA transcript (Lanier et al, 1986). However, populations of T3+ NK clones do have α and β transcripts that form a functional TcR (Ritz et al, 1985). Taken together, this evidence suggests that NK cells may arise from T-lineage precursors, but lysis may not be mediated by the TcR. With numerous secretory cytoplasmic granules observed in NK cells, the mechanism of killing closely resembles that of CTL, ultimately causing circular lesions in lysed target cell membranes (Young and Cohn, 1986). One mediator of NK cytolysis shares similarities with tumor necrosis factor, and has been termed Natural Killer Cytotoxic Factor (NKCF) (Marx, 1986). Additionally, a cytotoxic protein called cytolysin has been isolated from NK cell granules (Ortaldo et al, 1986).

Natural cytotoxic (NC) cells exhibit spontaneous killing about four times slower than NK cells (Ortaldo et al, 1986). In vitro, murine NC activity is measured against the fibrosarcoma WEHI-164.1 (Hackett et al, 1986). Murine NC activity is observed from birth through life, apparently declining with age. NC activity may be augmented with IL 3 and, like NK cells,

IL 2 (Lattime, 1986). NC and NK activities are enriched in fractions of large granular lymphocytes, suggesting that they may be closely related or even different stages of differentiation within a population of granulated lymphocytes. NC cytolysis appears to be mediated by tumor necrosis factor (Ortaldo et al, 1986).

LAK cells are capable of lysing a broad range of cells, including NK-insensitive targets, fresh autologous tumour cells, spontaneous murine adenocarcinoma cells, and most transplantable cell lines (Merluzzi, 1985). This activity is present from birth, when NK and IL 2-inducible NK are lacking (Merluzzi et al, 1986). LAK cells most resemble CTL phenotypically, with surface expression of variable levels of Thy1 and of asialo GM1 (Merluzzi, 1985). Also, like CTL, this activity is not spontaneous, requiring a three or four day incubation with IL 2 (Gray et al, 1985). Higher IL 2 concentrations are necessary to activate LAK cells than to enhance CTL activity (Merluzzi, 1985). Precursors of LAK cells appear to be distinct from precursors of CTL, expressing low or no T-cell markers (Ballas, 1986). In the human system, however, the LAK effector has been linked to T-cell lineage (Merluzzi et al, 1986).

A/3. THE IMMUNE RESPONSE TO PICHINDE VIRUS

A/3.1. The Humoral Immune Response to PV

Neutralizing serum antibodies are rarely detected in mice infected with certain members of the Arenavirus family (Parodi et al, 1970). Further studies with LCMV revealed the production of antiviral antibody that combines with viral antigen in circulation. Ultimately, the complexes lodge in smaller circulatory vessels such as the renal glomeruli, arteries, and choroid plexus of the brain, thus causing manifestations of immune complex disease (Oldstone et al, 1985). No neutralizing antibody response to PV has yet been detected in murine infections (Buchmeier et al, 1977).

A/3.2. The Cell Mediated Immune Response to PV

Strains of inbred mice inoculated with PV exhibit splenic NK and CTL activities in a cytotoxicity assay. This cell-mediated immune response has been observed in all strains so far examined. Murine splenic lymphocytes exhibit maximal CTL activity seven days after primary *in vivo* challenge with PV. An accelerated CTL response is observed four to five days after secondary infection of primed mice and after secondary *in vitro* restimulation of spleen cells with peritoneal exudate cells infected with homologous virus. Accordingly, enhanced NK

activity is observed three to four days after primary PV infection, and just one day after secondary *in vitro* or *in vivo* restimulation (Walker et al, 1984; Walker et al, 1985).

A/4. THE PURPOSE OF THIS STUDY

After the work of Walker *et al* (1984,1985) in this lab, it was thought that the PV system would be a good one from which to clone CTL and examine their relationship to nonspecific NK cells. It was hoped that with this model, the studies of Brooks (1983) that revealed reversible NK induction in CTL clones, could have been supported and extended. Further, it was hoped that this model would allow elucidation of CTL generation, fine target specificity, mechanism of action, and role *in vivo*, similar to the ongoing studies of cloned CTL against LCMV (Byrne et al, 1984). When such cloned lines failed to evolve, experiments were undertaken to examine the specificity of the anti-PV cell-mediated immune response more fully.

Previous studies have shown that murine anti-viral CTL may be restricted to either K or D ends of the H-2 complex (Zinkernagel, 1978). Therefore, the class I restriction of the anti-PV CTL response was mapped in H-2^b mice. In addition, the viral specificity of the anti-PV cell-mediated immune response was characterized

with a panel of PV mutant viruses which exhibited temperature sensitive defects in replication, and in the production of glycoprotein and/or nucleoprotein. With temperature sensitive (ts) mutant viruses, the target antigen specificity may be correlated with protein production and expression (Zinkernagel and Rosenthal, 1981). One mutant virus, ts 488, proved to be a superior target for cell-mediated lysis, and a greater stimulator of CTL. The immunogenicity of ts 488 combined with self-restricting molecules was then studied more fully in primary and secondary CTL responses. The ability of an immunogenic temperature sensitive mutant virus of wild type PV in facilitating the cloning of anti-PV CTL is discussed.

B./ MATERIALS AND METHODS

B/1. CELL LINES

B/1.1. Suspended Cell Lines

YAC-1, a subline of the YAC lymphoma induced by Moloney leukemia virus in A/Sn strain mice (Kiessling et al, 1975), was used as a ^{51}Cr labelled target cell indicating murine NK activity. This line was maintained in RPMI 1640 medium (Gibco, Grand Island, NY.) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 10 mM L-glutamine, 0.075% (w/v) sodium bicarbonate, 10mM N-20hydroxyethyl piperazine-N'-2-ethanosulfonic acid (HEPES), 100 ug/ml streptomycin, and 100 U/ml penicillin.

B/1.2. Attached Cell Lines

The murine fibroblast cell lines LTA ($H\text{-}2^k$), PAK ($H\text{-}2^b$), and 5R ($H\text{-}2K^bD^d$) were used as targets in ^{51}Cr release assays. LTA is a subline of L cells generated from connective tissue of a C3H/An mouse (Earle, 1943). The PAK line was derived from 20-methyl-cholanthrene-induced tumours (Simrell and Klein, 1979). Baby hamster kidney cells (BHK-21) and African green monkey kidney cells (Vero) were obtained from the American Type Culture Collection (ATCC, Rockville, MD.).

These attached cell lines were maintained in alpha-minimal essential medium (MEM; Gibco) supplemented with 10% FCS, 10 mM L-glutamine, 0.075% (w/v) sodium

bicarbonate, 100 ug/ml streptomycin, and 100 U/ml penicillin.

B/2. VIRUSES

B/2.1. Pichinde Virus

Pichinde virus (PV) (Strain AN3739) used in these studies was originally isolated from the rodent Oryzomys albigularis which was trapped in the Columbian Pichinde Valley (Trapido and Sanmartin, 1971).

B/2.1.1. Propagation and Titration of Wild Type PV

PV was grown in BHK-21 cells according to Mifune et al (1971). Briefly, subconfluent monolayers of BHK-21 cells in 150 cm^2 tissue culture flasks (Nunclon, Denmark) were inoculated at a multiplicity of infection (MOI) of 0.1 plaque-forming units per cell. Culture supernatants were harvested on ice at 48 and 72 hours after infection. The virus containing supernatant was centrifuged at 1500 rpm for ten minutes, and stored at-70°C.

The plaque assay method of Mifune et al (1971) was used to titer the virus in plaque-forming units (pfu) per ml. 0.4 ml of serial ten-fold dilutions of the virus-containing supernatants were inoculated onto subconfluent monolayers of Vero cells in 60 mm tissue

culture dishes (Falcon). The supernatant was aspirated after a sixty minute incubation in a humidified 37°C incubator. An overlay of 4 mls of maintenance medium containing equal parts of 1.75% (w/v) agar (Difco, Detroit, MI.) and 2X Hanks's Balanced Salt Solution supplemented with 20% FCS, 20 mM L-glutamine, 0.150 (w/v) sodium bicarbonate, 20 mM HEPES, 200 ug/ml streptomycin, 200 U/ml penicillin, 2% (v/v) BME amino acids (Gibco), and 2% (v/v) 100X BME vitamins (Gibco) was then added. Three days after incubation at 37°C, a second overlay of 4 mls of maintenance medium containing 1% (v/v) neutral red dye (Gibco) was added. Plaques were counted 24 hours after incubating at 37°C.

B/2.1.2. Preparation, Propagation, and Titration of Conditionally Lethal Mutants of PV

Temperature sensitive (ts) mutants of PV, were generously provided by Dr. W.E. Rawls. They were generated according to Vezza et al (1977). Briefly, confluent monolayers of BHK-21 cells were inoculated with wild type PV at an MOI of 1 in Dulbecco's modified Eagle Medium supplemented with 10% FCS and 0.4 mM 5-fluorouracil. After 48 hours of incubation, the virus-containing supernatants were harvested, centrifuged at 1500 rpm for 10 minutes, and plated on Vero cells at 34°C. Several plaques were picked, and each used to

inoculate duplicate 25 cm^2 flasks of BHK-21 cells. One flask was incubated at the permissive temperature of 34°C, and the other at the non-permissive temperature of 39.5°C. The virus-containing supernatant was harvested after 48 hours and titred at both temperatures by the plaque assay on Vero cells as described. Plaques were picked from mutants exhibiting a 39.5/34°C efficiency of plating (EOP) value of less than 0.01, and were used to infect subconfluent monolayers of BHK-21 cells for propagation at 34°C. Like the method described for wild type PV, virus-containing supernatants were harvested 48 and 72 hours after infection, and titred on Vero cell monolayers at 34 and 39.5°C. Only harvests with an EOP of less than 0.01 were used.

B/3. GENERATION OF CELL-MEDIATED IMMUNE RESPONSES

B/3.1. Primary Cell-Mediated Immune Responses

Mice purchased from Jackson Laboratories (Bar Harbour, ME.) were used between six and nine weeks of age. They were supplied with Purina mouse chow and water ad libitum, and housed in polycarbonate wire topped cages. Mouse strains used were C57Bl/6 ($H\text{-}2^b$), B10.A (5R) ($H\text{-}2K^bD^d$), and B10.A.(2R) ($H\text{-}2K^dD^b$) (Klein, 1975).

Mice were primed by intravenous (i.v.) inoculation with $6x10^6$ pfu of PV. Spleens were excised seven to

nine days following immunization. A single cell suspension was prepared by passing the spleens through a wire mesh grid.

B/3.2. Secondary Cell-Mediated Immune Responses in vitro

Five weeks after i.v. inoculation with PV, murine spleens were removed aseptically. A single cell suspension of responders was prepared by passing the spleens through a wire mesh grid.

Stimulator cells were obtained by peritoneal lavage with PBS of mice injected intraperitoneally with sterile thioglycollate broth (Difco, Detroit, MI.) two to four days previously. Stimulators were irradiated with 1500 R from a Cs source, then infected at 37°C at an MOI of 0.1 for 45 minutes.

Cells were generally cultured at a responder:stimulator ratio of 10:1 in RPMI medium supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UH), 10mM L-glutamine, 0.075% (w/v) sodium bicarbonate, 10mM HEPES, 100 ug/ml streptomycin, 100 U/ml penicillin, and 5×10^{-5} M 2-mercaptoethanol, with cells at a density of approximately 1×10^{6} per ml. Cultures were incubated for five days in a humidified 37°C incubator before harvesting effector lymphocytes.

B/4. ASSAY FOR CELL-MEDIATED CYTOTOXICITY

Cytotoxicity was measured against ^{51}Cr-labelled targets. For virus-specific lysis, targets were infected with PV at an MOI of approximately 10, 24 hours before the assay. Natural killer activity was measured against YAC cells. To label, targets were suspended in 1 ml of MEM with 300 uCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA.) for 90 minutes. Subsequently, targets were washed four times in MEM, and suspended at $1x10^6$ cells/ml. Effector cells from primary immunized mouse spleen or secondary in vitro cultures were generally set at $7x10^6$, or as indicated.

50 ul of the target cell suspension was placed in wells of 96 well microtiter plates (Nunclon, Denmark). Effectors were added at typical effector:target ratios of 40:1, 12:1, and 4:1, or as indicated, and assayed in duplicate. Wells were filled with medium. ^{51}Cr release from a target in the experimental wells was compared with that released in control wells. The minimum release control was determined by incubating targets in medium alone. The maximum release control was determined by incubating targets in 1 N hydrochloric acid (HCl). Control wells were also assayed in duplicate.

After incubating the assays for specific lytic activity for seven and a half hours, and assays for

natural killer activity for four hours, 100 ul of the supernatant was removed from each well and pipetted into 6x50 mm glass tubes (Kimble) for counting in a gamma counter.

The number of cpm released from each of the two test wells were not different by more than five per cent of the mean. Results were repeated in at least three concordant assays. The average number of cpm released from duplicate wells was used in calculations.

Spontaneous release was calculated by:

$$\frac{\text{Average minimum cpm (minimum control)}}{\text{Average maximum cpm (HCl control)}} \times 100$$

Acceptable values were generally less than 25%.

Per cent specific lysis was calculated by:

$$\frac{\text{Average sample cpm} - \text{Average minimum cpm}}{\text{Average maximum cpm} - \text{Average minimum cpm}} \times 100$$

B/5. LIMITING DILUTION ASSAY FOR CTL PRECURSOR FREQUENCY

B/5.1. Limiting Dilution Culture

The frequency of CTL precursors was determined by limiting dilution assay similar to the method of Askonas et al (1982). Stimulator and responder cells were prepared in the same method as for bulk secondary *in vitro* cultures. In addition to responder splenocytes and PV-infected stimulators, PV memory cells irradiated

with 1200 R from a Cs source were used as feeder cells in order to satisfy the requirements for exogenous helper factors.

Specifically, five serial 1.5-fold dilutions of responder cells were cultured with $1x10^5$ virus-infected, 1500 R irradiated peritoneal exudate cells as stimulators, and $1.5x10^5$ feeder cells, in flat bottom microtiter plates in a final volume of 0.2 ml RPMI supplemented with 10% heat-inactivated FCS (Hyclone), 10mM L-glutamine, 0.075% (w/v) sodium bicarbonate, 10 mM HEPES, 100 ug/ml streptomycin, 100 U/ml penicillin, and $5x10^{-5}$ M 2-mercaptoethanol Additionally, control wells were prepared with $1x10^5$ stimulator cells plus $1.5x10^5$ feeder cells alone. Usually, 16 or 24 replicates, as indicated, were prepared at each dilution for assay of cytolysis. Aluminium foil-wrapped plates were incubated for five days at 37°C in a humidified atmosphere, and cytotoxicity was determined in a ^{51}Cr release assay.

B/5.2. Limiting Dilution ^{51}Cr Release Assay

The assay method was similar to that of Askonas et al (1982). Targets were infected, labelled, and washed as described above. $1x10^4$ targets in 0.05 ml of medium were added to each well. Plates were incubated for seven and a half hours at 37°C, then 100 ul of supernatant was removed from each well for counting.

Typically, experimental wells were scored positive if the number of cpm released was at least three standard deviations greater than the mean number of cpm released from control wells, or as indicated. Finally, CTL precursor frequency was determined by the Maximum Likelihood Method of Porter and Berry (1964). These calculations are described in Appendix A, on page 95.

B/6. ^{3}H THYMIDINE INCORPORATION ASSAY FOR CELL PROLIFERATION

Spleen cells from mice infected with PV five weeks prior to use were cultured with virus-infected, 1500 R irradiated peritoneal exudate cells in flat bottom microtiter wells (Nunclon). Usually six replicates were prepared with $3x10^{5}$ responder spleen cells combined with $3x10^{4}$ stimulator cells. Cultures were incubated at 37 C, and were labelled with 5 uCi of ^{3}H Thymidine for the final 18 hours of culture. Subsequently, the contents of the wells was harvested onto glass fiber strips with a Mini-Mash harvester. Finally, 5 mls of ACS scintillation fluid were added to each filter for detection of radioactivity in a Beckman scintillation counter.

C./ RESULTS

C/1. H-2 SPECIFICITY OF THE ANTI-PV CELLULAR IMMUNE RESPONSE

Cytotoxic T lymphocyte (CTL) responses generally exhibit dual specificity for a particular antigen combined with Class I H-2 antigens (Nabholz, 1983). The latter are glycoprotein "self" molecules that are encoded by the major histocompatibility complex (MHC) and displayed on target cell membranes. Studies have shown that the antiviral immune response may be mediated predominantly by either the K or D class I loci encoded by host H-2 (Zinkernagel, 1978). In point of fact, the immune mediated pathology of LCMV appears to be predominantly linked to the H-$2D^b$ locus (Zinkernagel et al, 1985). The following studies attempt to map the genetic end restriction of the H-2^b anti-PV cell-mediated immune response using cytotoxicity assays, and limiting dilution assays.

C/1.1. End-Restricted Cytotoxicity

In order to map the end-restriction of the primary anti-PV cytotoxic response, effector spleen cells from three inbred mouse strains were assayed for cytotoxicity against three different target cells. This data is displayed in Table 1.

TABLE 1

H-2 END RESTRICTION OF ANTI-PV CTL

EFFECTOR[a]	E:T[b]	% SPECIFIC 51CR RELEASE FROM TARGET CELLS[c]					
		PAK ($H\text{-}2K^bD^b$)		5R ($H\text{-}2K^bD^d$)		LTA ($H\text{-}2K^dD^b$)	
		UN	PV	UN	PV	UN	PV
C57BL/6 ($H\text{-}2K^bD^b$)	40:1	30	73	13	64	14	9
	12:1	23	58	8	42	8	7
	4:1	18	23	5	20	2	2
B10.A(2R) ($H\text{-}2K^dD^b$)	40:1	31	40	12	13	5	4
	12:1	27	31	9	10	6	2
	4:1	35	12	6	5	4	0
B10.A(5R) ($H\text{-}2K^bD^d$)	40:1	32	70	12	58	7	2
	12:1	28	53	10	24	5	4
	4:1	26	24	7	15	5	0

a Groups of 3 mice were infected with PV 7 days prior to the assay.

b Effector to target cell ratio as indicated.

c Assay time 7.5 hours, spontaneous release <30%.

Neither uninfected target cells nor histoincompatible LTA cells were lysed by anti-PV CTL. B10.A(2R) effectors exhibited a low level of lysis against the PV-infected, D^b compatible PAK target cell. A high level of lysis of PV-infected PAK target cells was observed by H-$2K^b$ compatible B10.A(5R) effectors and by syngeneic C57Bl/6 effectors. In addition, PV-infected 5R targets were lysed to a similar extent by H-$2K^b$ compatible C57Bl/6 effectors and by syngeneic B10.A(5R) effectors.

Since maximum lysis was observed when effector and target were compatible at the H-$2K^b$ locus, the primary anti-PV response appears to be predominantly restricted by the K end of the H-2^b complex.

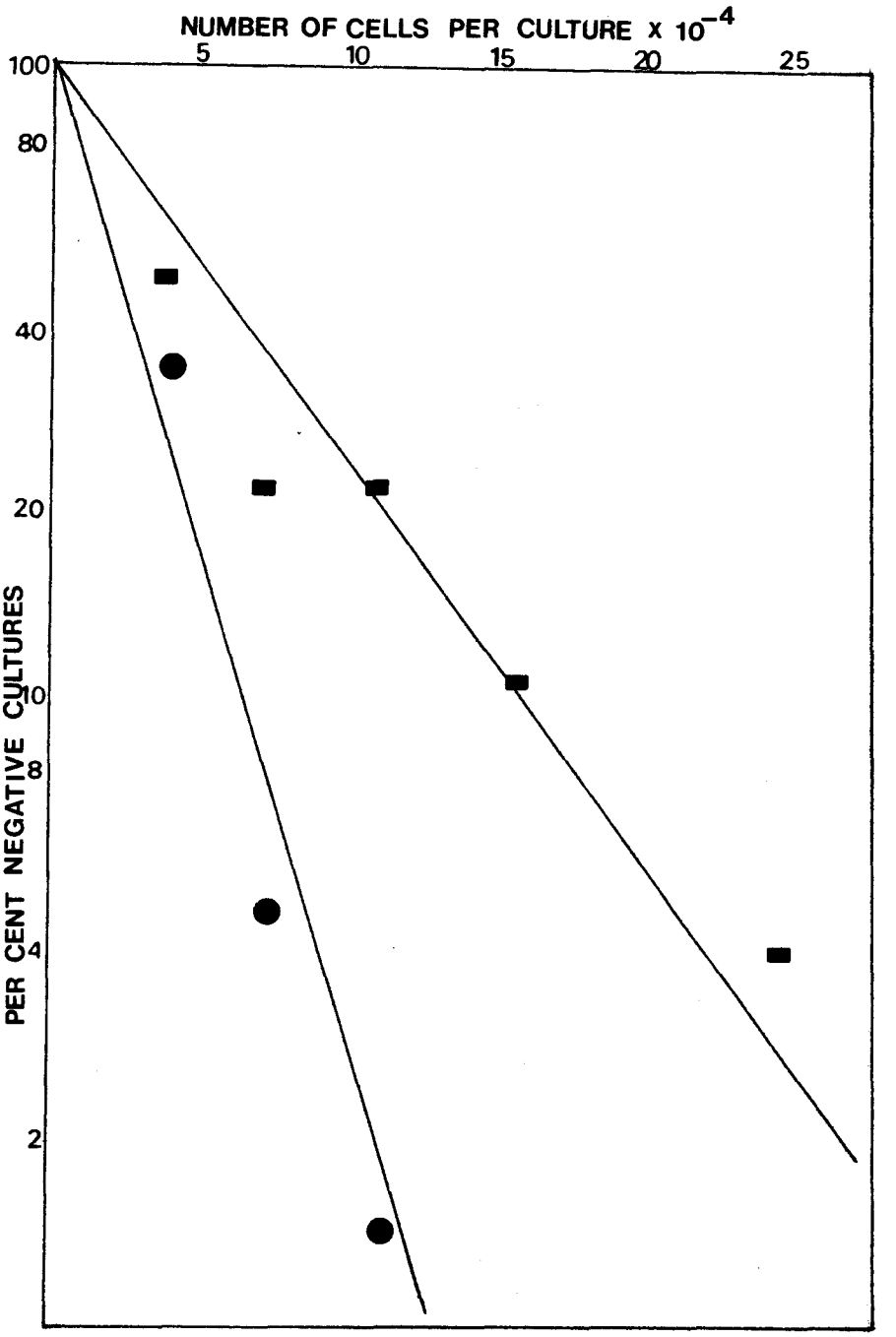
C/1.2. Limiting Dilution Analysis for End-Restricted CTL Precursor Frequency

To further map the end restriction of the anti-PV CTL response, a limiting dilution assay (LDA) was used to determine the frequency of K^b-restricted CTL precursors (CTLp) in a secondary *in vitro* anti-PV response. H-2^b memory effectors were cultured at limiting dilution, and assayed for cytotoxicity five days later against PV-infected, syngeneic PAK targets, and K^b compatible 5R targets. As seen in Figure 1, the frequency of CTLp restricted to the K-end is approximately half the number of CTLp specific for

completely syngeneic target cells, using the Maximum Likelihood Method of Porter and Berry (1964) to calculate CTLp frequency. The frequency of CTLp generated in C57Bl/6 PV memory spleens against completely syngeneic PAK target cells infected with homologous virus was 1/31,508, with the 95% confidence interval ranging from 1/23,123 to 1/42,932. Against 5R target cells compatible only at the K locus, the CTLp frequency is 1/69,536, with the 95% confidence interval ranging from 1/54,469 to 1/88,770. Thus, in determining the frequency of K-end restricted CTLp, the same preponderance of K-end restricted killers observed in bulk cultures was not observed under limiting dilution conditions. These results show that only about half of the CTL precursors in memory spleens are restricted by the K end of the $H\text{-}2^{b}$ complex. In contrast to these results, the cytotoxic T cell response to LCMV in mice was found to be predominantly regulated by the H-2D region (Zinkernagel et al, 1985b).

FIGURE 1: LIMITING DILUTION ASSAY FOR K^b-RESTRICTED CTL PRECURSOR FREQUENCY

C57Bl/6 mice were infected with PV approximately five weeks before the assay. Spleens were excised and cocultured with stimulator and feeder cells for five days, and then assayed for cytotoxicity against PV-infected H-2$K^b D^b$ PAK (●) and H-2$K^b D^d$ 5R (■) targets as described earlier. Spontaneous release of ^{51}Cr was determined by incubating target cells with stimulator and feeder cells alone. Wells were scored as positive for cytotoxicity if the counts released from a well were greater than or equal to 3 SD above spontaneous release. Determination of frequency was made with 24 replicate cultures at each responder dilution. Spleen cells did not cause significant lysis of uninfected PAK or 5R target cells. The precursor frequency against PAK-PV was 1/31,508, with a 95% confidence interval of 1/23,123 to 1/42,932. The precursor frequency against 5R-PV was 1/69,536, with a 95% confidence interval of 1/54,469 to 1/88,770. The correlation coefficient of the line, calculated as the x^2 value, was 3.28 against PAK targets and 1.72 against 5R targets.


NUMBER OF CELLS PER CULTURE x 10^{-4}
5
10
15
20
25
100
80
40
20
10
8
4
2
PER CENT NEGATIVE CULTURES

C/2. VIRUS SPECIFICITY OF THE ANTI-PV CELLULAR RESPONSE

C/2.1. The Primary Cytotoxic Response

C/2.1.1. Temperature Sensitive Mutants of PV as CTL Targets

One approach to examine the target specificity of anti-viral CTL is to study temperature sensitive (ts) mutants which variably produce viral proteins and allow correlation of lysis with protein expression (Zinkernagel and Rosenthal, 1981). In order to examine the target specificity of anti-PV CTL, a panel of temperature sensitive (ts) mutants of PV were assayed for their ability to serve as targets for CTL from mice immunized with wild type (wt) PV. Each mutant had been characterized with respect to production and expression of nucleoprotein (NP) and glycoprotein (GPC) expression as measured by polyacrylamide gel electrophoresis and indirect immunofluorescence.

As can be seen in Table 2, the temperature sensitive mutants were divided into five groups according to their characteristic surface fluorescence and protein phenotype. Normal protein phenotypes were observed at the permissive temperature for all groups of mutants except those in group E, where expression of NP and GPC was reduced at 34°C as well as 39.5°C. The ability of these mutant viruses to act as CTL targets was studied for correlation with protein expression.

Figures 2,3, and 4 which follow, illustrate specific lysis of these ts mutant viruses.

TABLE 2: PROTEIN PHENOTYPE OF TEMPERATURE SENSITIVE MUTANTS OF PICHINDE VIRUS (After Prakash, Master's Thesis, 1986)

Group	Ts Mutants	39°C Protein Phenotype[a]		39°C Surface Fluorescence[b]
		NP	GPC	
A	274,488	normal	normal	normal
B	1,3,5,538,939	reduced	absent	absent
C	9,13,908	normal	increased	absent
D	2,10	reduced	reduced	reduced
E	11,454	reduced	reduced	reduced

a Proteins detected by polyacrylamide gel electrophoresis.

b Proteins detected by indirect immunofluorescence.

FIGURE 2: LYSIS OF 5R TARGET CELLS INFECTED WITH TS 5, TS 13 OR WT PV

PV immune spleen cells from C57Bl/6 mice were assayed seven days after primary immunization for CTL activity against 5R target cells which were uninfected (•••), or infected with either ts 5 (■), or with ts 13 (●) from groups B and C respectively, or with wt PV (▮). Assays were incubated at 34 for 7.5 hours or 39.5 C for six hours, as indicated. Spontaneous release was less than 25%. Effector to target ratios assayed were 40:1, 12:1 and 4:1.

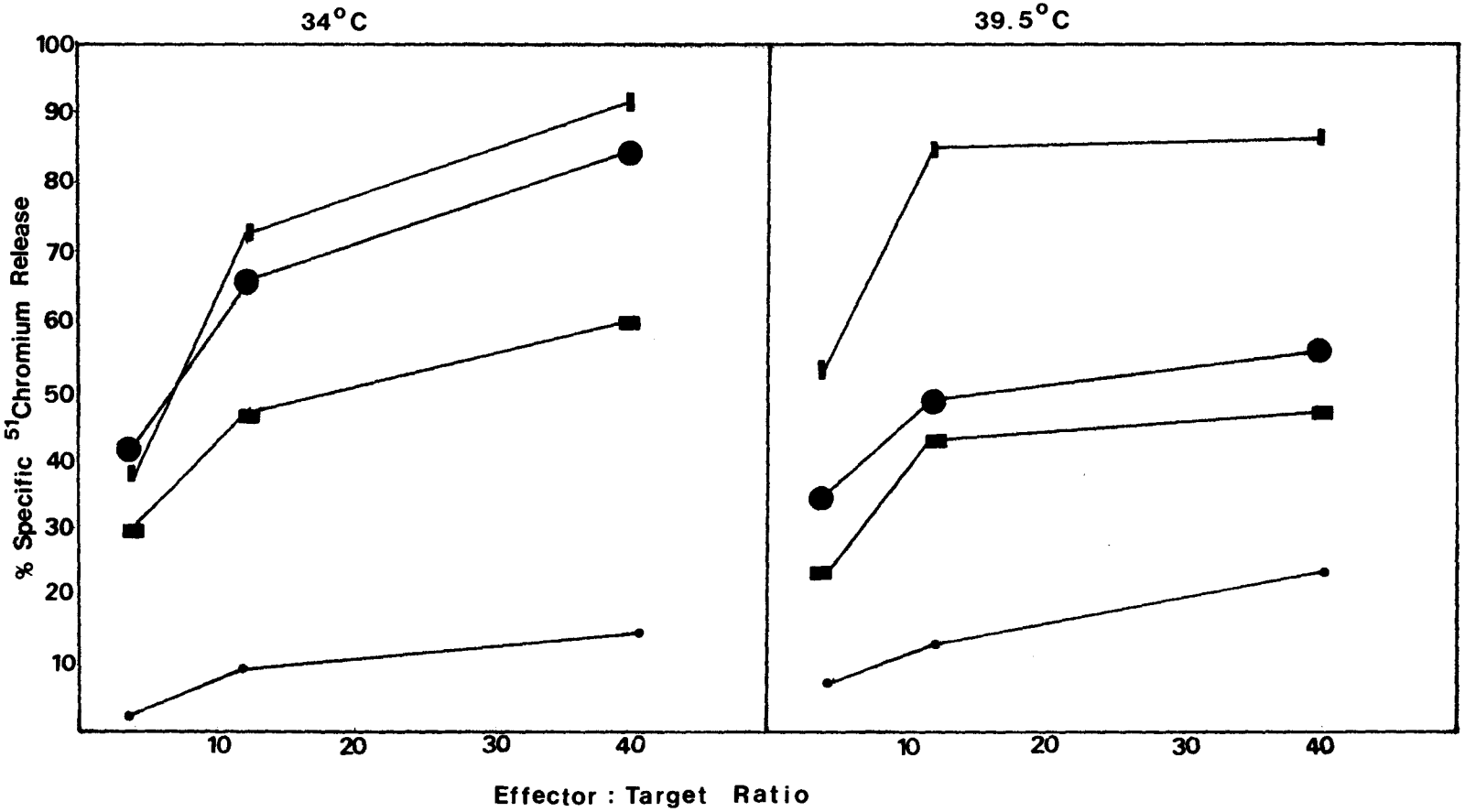

34°C
39.5°C
% Specific 51Chromium Release
100
90
80
70
60
50
40
30
20
10
10
20
30
40
10
20
30
40
Effector : Target Ratio

FIGURE 3: LYSIS OF 5R TARGET CELLS INFECTED WITH TS 2.2, TS 908 OR WT PV

Primary C57Bl/6 PV immune spleen cells were assayed for their ability to lyse 5R targets which were uninfected (•••), or infected with ts 2.2 (■) from group C or ts 908 (●) from group D, or wt PV (▮). Assays were incubated at either the permissive temperature of 34 C for 7.5 hours, or the non-permissive temperature of 39.5 C for six hours, as indicated. Spontaneous release was less than 25%. Effector to target ratios assayed were 40:1, 12:1, and 4:1.

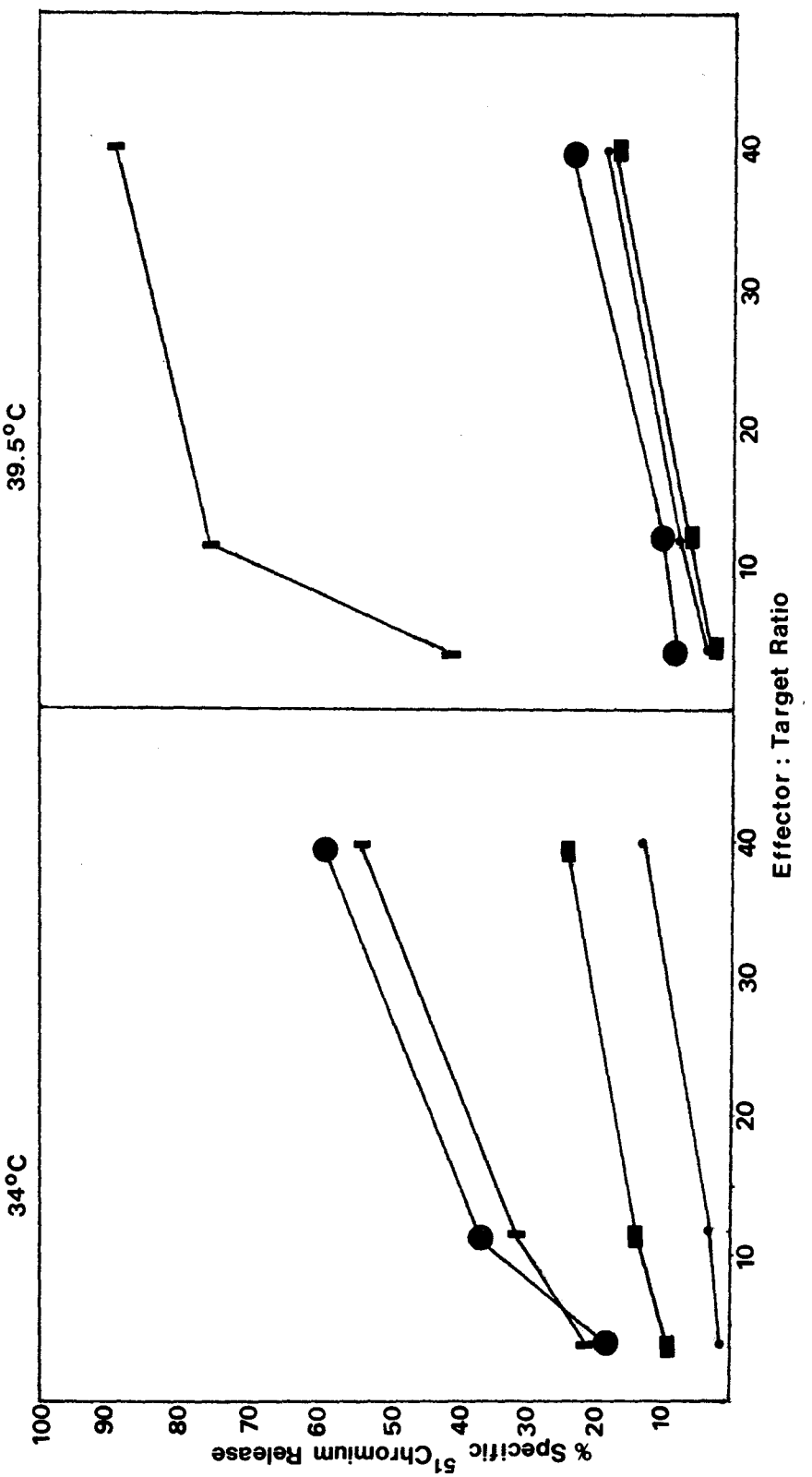

34°C
39.5°C
% Specific 51Chromium Release
100
90
80
70
60
50
40
30
20
10
10
20
30
40
Effector : Target Ratio

FIGURE 4: LYSIS OF 5R TARGET CELLS INFECTED WITH TS 11, TS 454 OR WT PV

C57Bl/6 mice were immunized with PV i.v. seven days before assaying spleen cells for anti-PV CTL activity in a ^{51}Cr Release Assay. Effectors were incubated with 5R target cells which were uninfected (•••) or infected with wt PV (▮),or the group E viruses ts 11 (●), or ts 454 (■). Assay duration was 7.5 hours at 34 C, or six hours at 39.5°C, with temperatures as indicated. Spontaneous release was less than 25%. Effector to target ratios assayed were 40:1, 12:1, and 4:1.

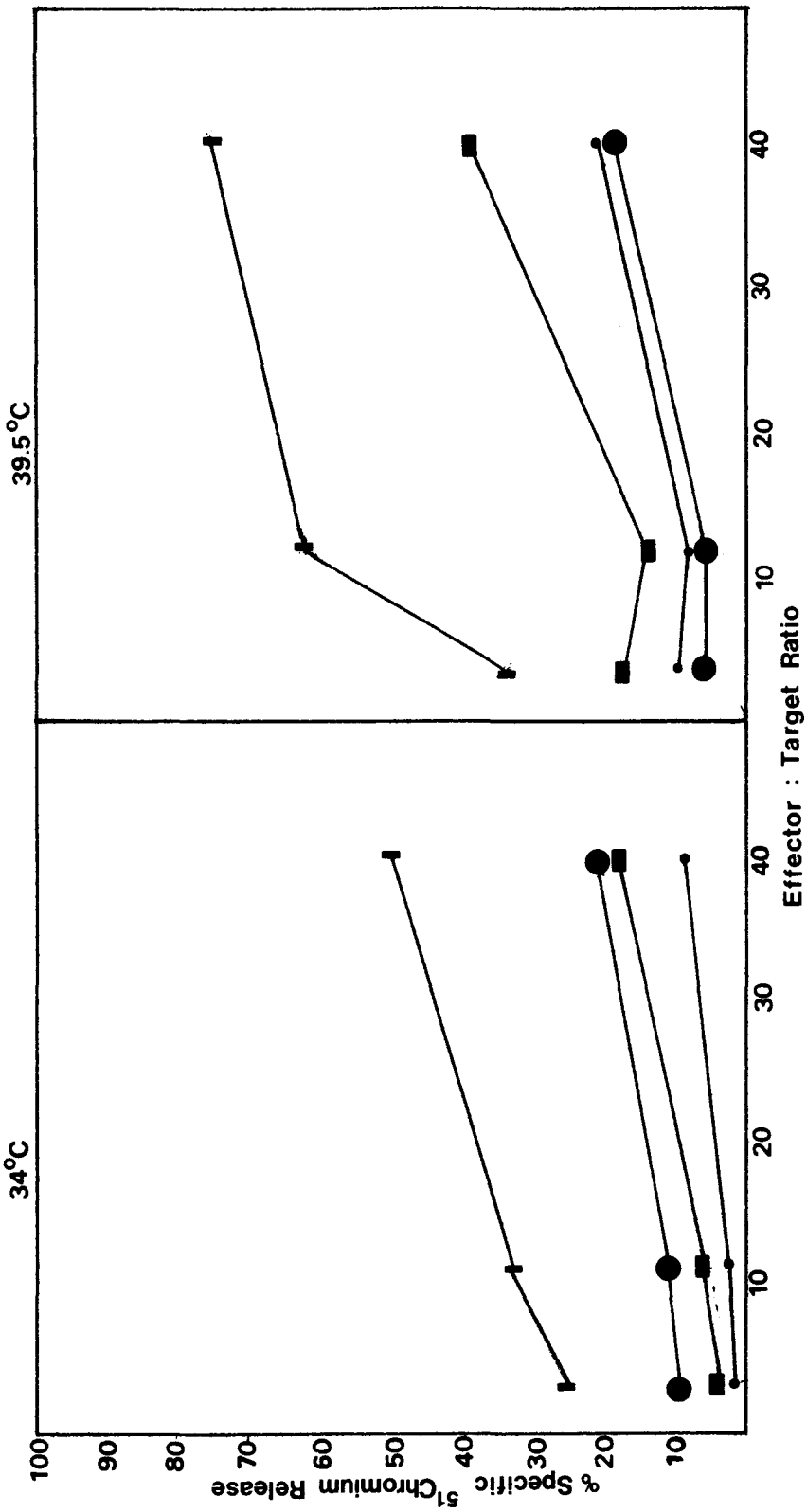
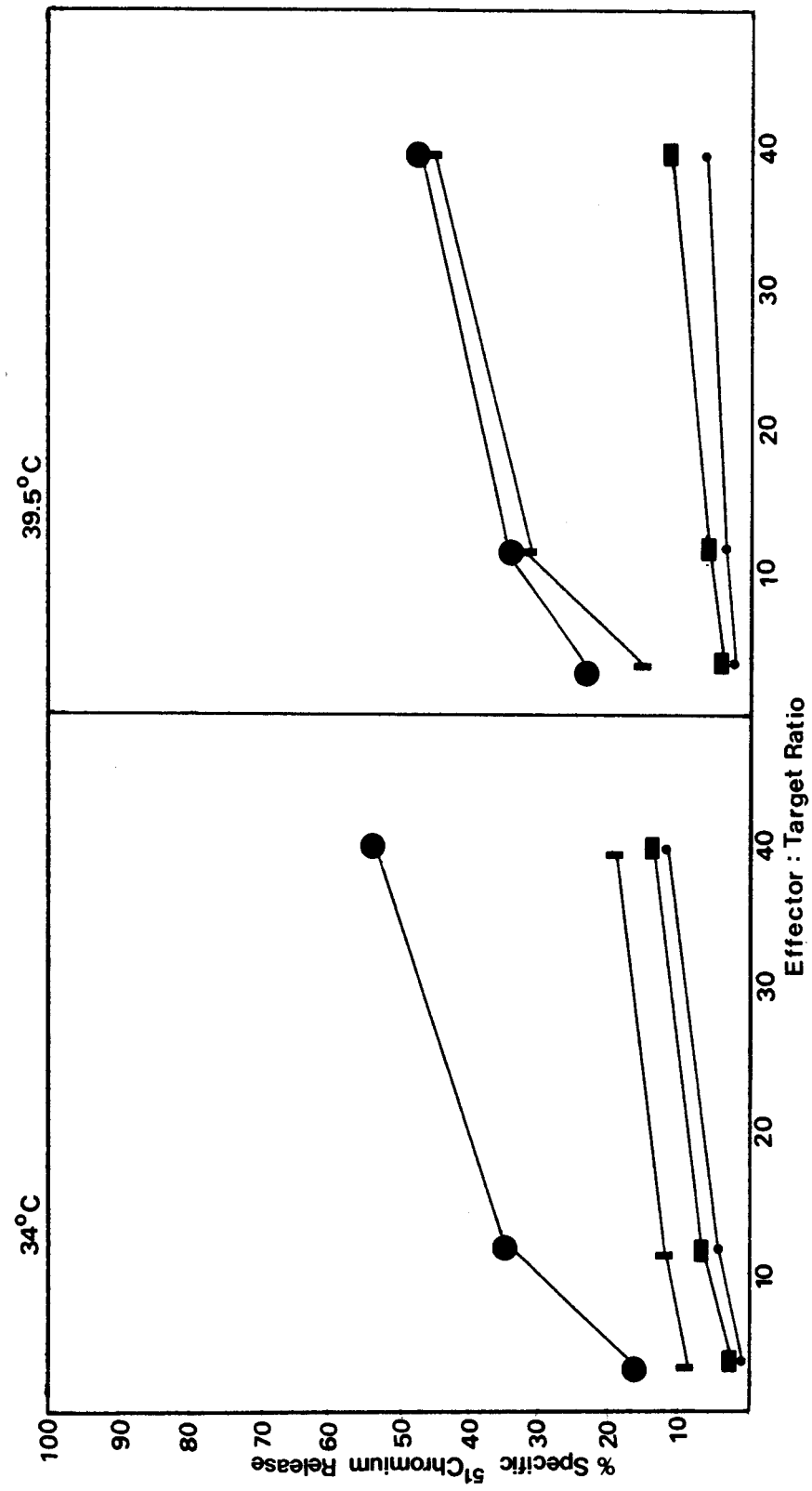

34°C
39.5°C
% Specific ^{51}Chromium Release
100
90
80
70
60
50
40
30
20
10
10
20
30
40
Effector : Target Ratio

FIGURE 5: LYSIS OF 5R TARGET CELLS INFECTED WITH TS 274, TS 488, OR WT PV

Spleen cells were assayed for cytotoxicity seven days after primary i.v. immunization with PV. Effectors were incubated with 5R target cells which were uninfected (•••), or infected with wt PV (▮), or the group A viruses ts 274 (■), or ts 488 (●). Assay duration was 7.5 hours at 34°C, or 6 hours at 39.5°C, with temperatures as indicated. Spontaneous release was less than 25%. Effector to target ratios assayed were 40:1, 12:1, and 4:1.


34°C
39.5°C
% Specific 51Chromium Release
100
90
80
70
60
50
40
30
20
10
10
20
30
40
Effector : Target Ratio

In all assays, low levels of lysis of uninfected 5R targets were observed. Figure 2 shows specific lysis of 5R targets infected with ts 5, ts 13, or wt PV. At the permissive temperature, ts 5, a member of group B, was lysed moderately compared to wt PV. In contrast, ts 13, a member of group C, was lysed at a level similar to wt PV. At the non-permissive temperature, both ts mutants 5 and 13 showed intermediate levels of lysis, with the latter remaining a slightly superior target.

Figure 3 illustrates specific lysis of 5R targets infected with ts 2.2, ts 908, or wt PV. At 34°C ts 908, a member of group C, was lysed in excess of wt PV. Low levels of lysis of ts 2.2, a member of group D, were evident. An increase in temperature to 39.5°C abrogated lysis of both targets. Since ts 908 failed to display surface protein in indirect immunofluorescence at the non-permissive temperature, its lack of CTL target formation may correlate with a lack of GP1 and GP2, surface glycoprotein cleavage products of GPC.

Specific lysis of 5R targets infected with ts 11 or ts 454 with that of wt PV is illustrated in Figure 4. Both ts mutants are assigned to group E on the basis of their protein phenotypes. At 34°C, targets infected with either ts 11 or 454 were lysed at very low levels. At 39.5°C, lysis of ts 11 decreased further, while lysis of ts 454 increased only moderately above background

levels.

Figure 5 displays specific lysis of 5R targets infected with wt PV or with a member of group A, either ts 274 or ts 488. Targets infected with ts 274 were lysed just slightly above background levels at both 34°C and 39.5°C. Conversely, targets infected with ts 488 were lysed as well as those infected with wt PV at 39.5°C, and to a greater extent at 34°C.

These results offer no clear correlation between protein expression and lysis of ts mutant viruses. The results suggest that the ability to act as a target may be loosely related to the surface expression of viral protein, and more specifically to surface glycoproteins GP1 and/or GP2 as suggested by the results of ts 908.

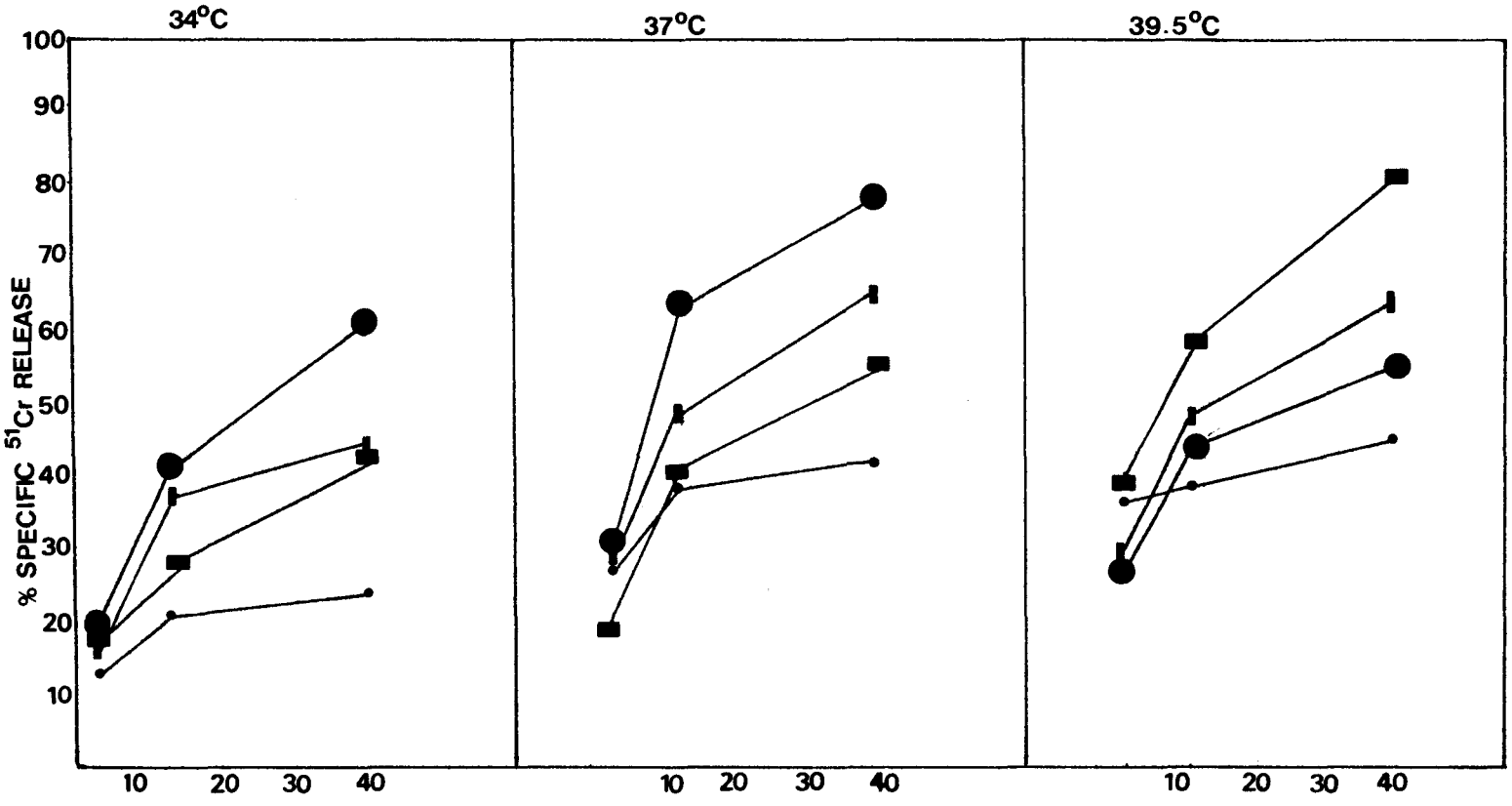
C/2.1.2. Wild Type Versus Temperature Sensitive Mutant 488 as a CTL Target

Preliminary observations, illustrated in Figure 5, indicated that target cells infected with the group A virus ts 488, could be lysed at least as well or to a greater extent than those infected with wt PV. This led to closer examination of ts 488 in both primary and secondary anti-PV immune responses. CTL from spleens of PV-infected C57Bl/6 mice were assayed against PAK and 5R cells which were infected with wild type (wt) or

temperature sensitive mutant 488 (ts 488) PV, at the temperature permissive to the mutant (34°C), non-permissive to the mutant (39.5°C), and at physiologic temperature (37°C). In PAK targets, CTL exhibited maximal lysis against wt-infected cells in all three assay conditions. Conversely, 5R targets infected with ts 488 were lysed more effectively at 34 and 37°C. At the non-permissive temperature, however, wt-infected 5R cells provided a superior CTL target. This data is depicted in Figure 6.

FIGURE 6: COMPARISON OF TS 488- AND WT PV-INFECTED PAK AND 5R TARGETS IN A PRIMARY ANTI-PV CYTOTOXIC RESPONSE

C57Bl/6 mice were inoculated with wt PV seven days before assaying spleen cells in a ^{51}Cr release assay with PAK ($H\text{-}2K^bD^b$) cells infected with either wt (▮) or ts 488 (• •) PV, or 5R cells ($H\text{-}2K^bD^d$) infected with either wt (■) or ts 488 (●). Cells were assayed at the permissive temperature (34°C), the non-permissive temperature (39.5°C), and at physiologic temperature (37°C). The spleen effector to target cell ratios assayed were 40:1, 12:1, and 4:1. The duration of the assay was 7.5 hours, and spontaneous release was less than 25%. Uninfected PAK and 5R targets were not lysed.


34°C
37°C
39.5°C
100
90
80
70
60
50
40
30
20
10
% SPECIFIC 51Cr RELEASE
10 20 30 40
10 20 30 40
10 20 30 40
EFFECTOR TO TARGET RATIO

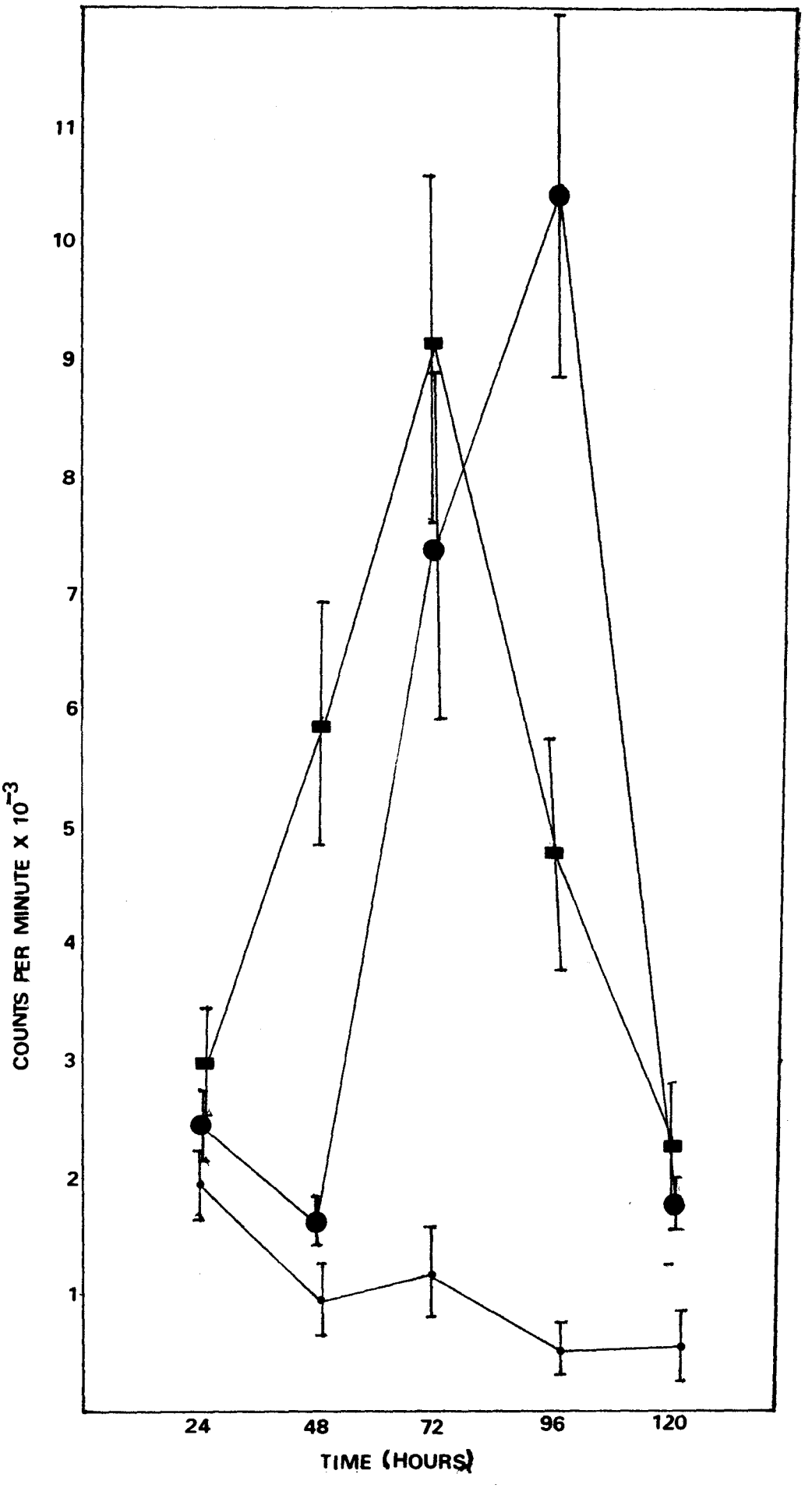
C/2.2. The Secondary Cytotoxic Response

C/2.2.1. Wt versus Ts 488 Proliferation Time Course

The ability of ts 488 to stimulate memory spleen cells was examined by cellular proliferation as measured by ^{3}H-Thymidine incorporation. Proliferation was assessed at daily intervals for microcultures of memory spleen cells restimulated with peritoneal exudate cells infected with either wt or ts 488 PV. As illustrated in Figure 7, restimulation with ts 488 induced cellular proliferation comparable to that induced by restimulation with wt PV. Maximum proliferation with the latter, however, occured approximately 24 hours earlier than with the former.

FIGURE 7: TIME COURSE OF CELLULAR PROLIFERATION IN A SECONDARY CYTOTOXIC RESPONSE RESTIMULATED BY WT OR TS 488 PV

Mice were infected with wt PV five weeks before excising the spleen for coculture at 37°C with antigen presenting cells which remained uninfected (•), or were infected with either wt (■) or ts488 (●) PV. Cellular proliferation was assessed at daily intervals by labelling cultures for 18 hour periods with ^{3}H Thymidine as described in Materials and Methods. Results are displayed as the mean of six microcultures ± the standard deviation.


11
10
9
8
7
6
5
4
3
2
1
COUNTS PER MINUTE X 10^{-3}
24
48
72
96
120
TIME (HOURS)

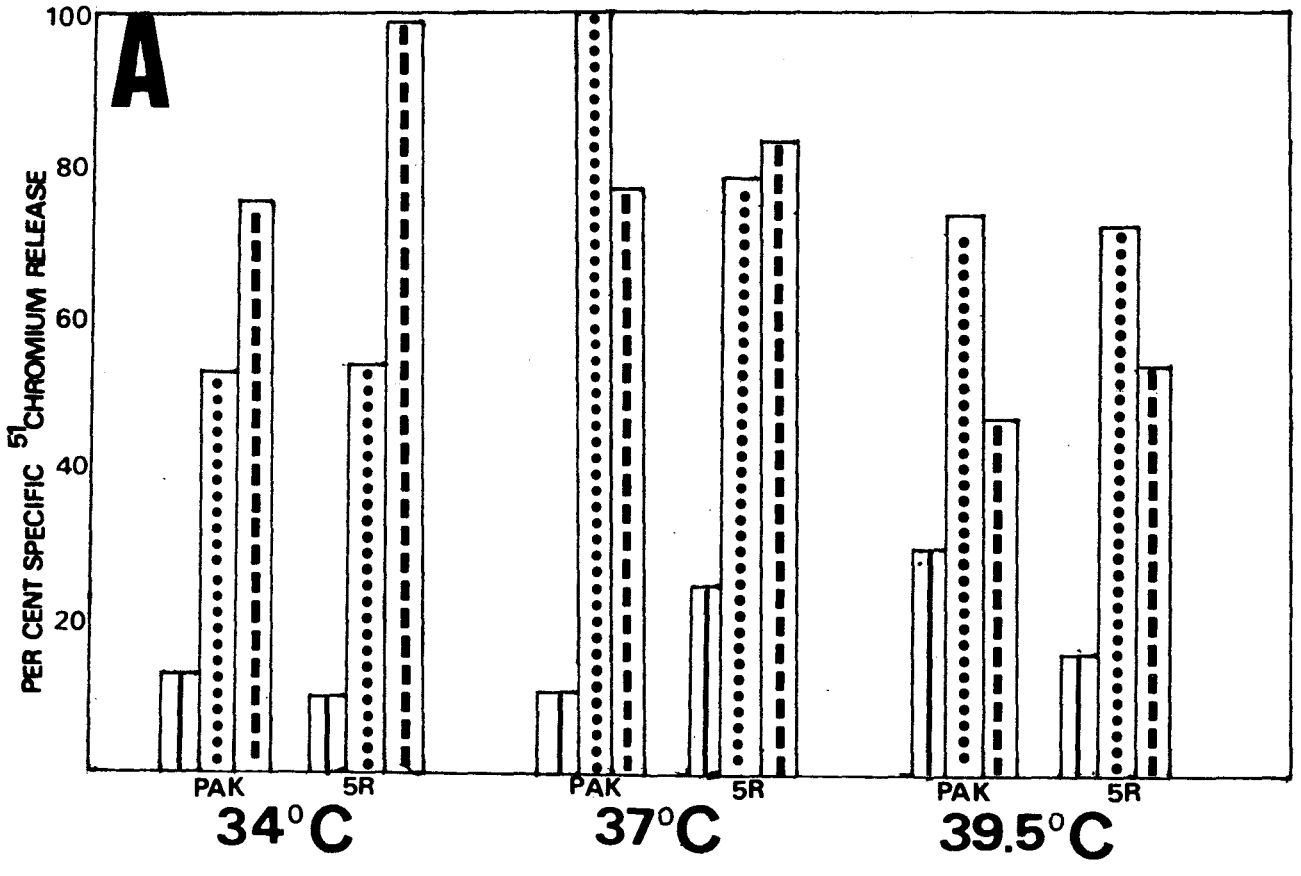
C/2.2.2. Comparison of Ts 488 with Wt PV as Restimulators and as Targets

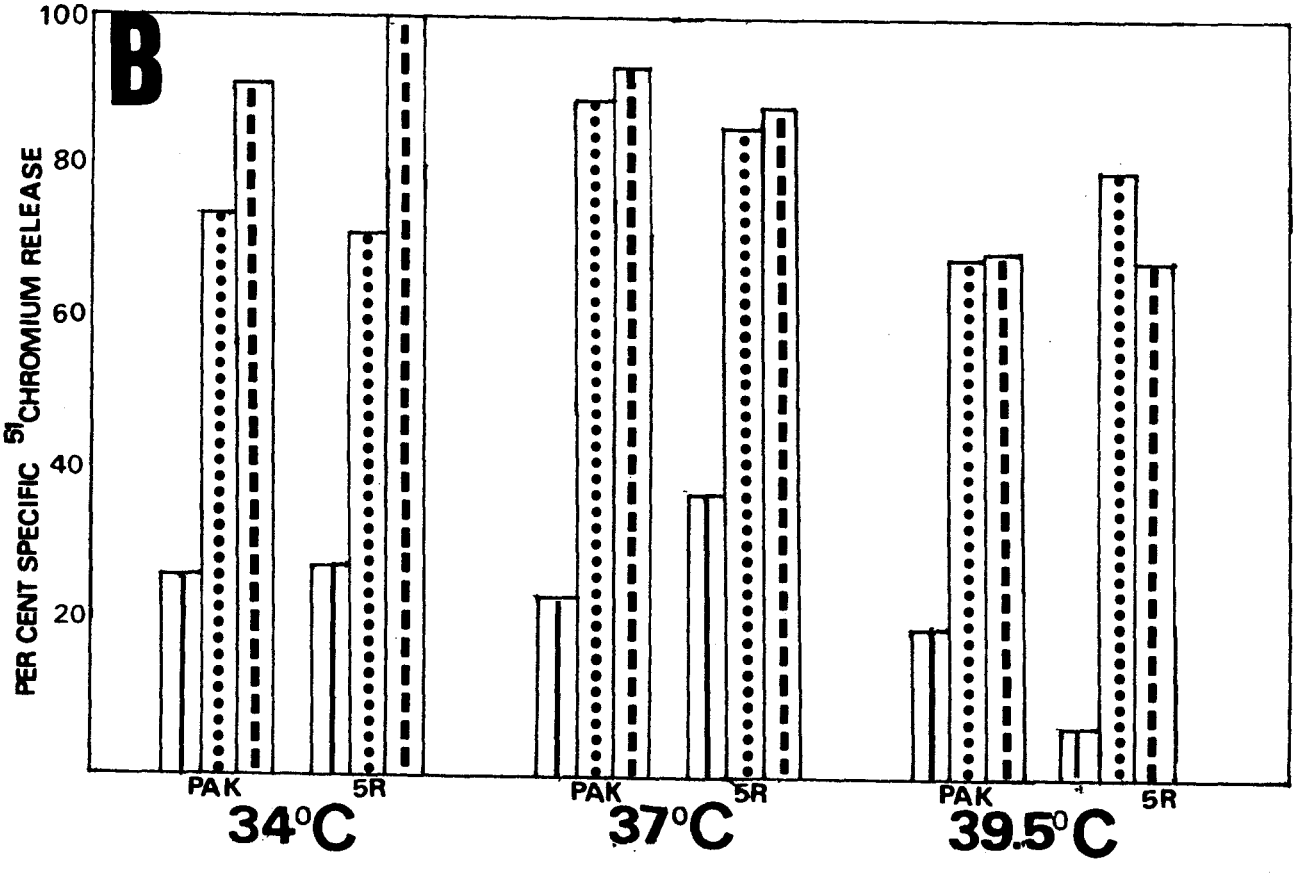
In order to compare the ability of ts 488 and wt PV for their ability to restimulate wt-primed C57Bl/6 spleen cells, secondary *in vitro* cultures were prepared as described, with antigen presenting cells infected with either wt or ts 488 PV. In either case, cultures were maintained at physiologic temperature, 37°C. After incubating for five days, effector cells were assayed against PAK or 5R target cells infected with either wt or ts488 PV at 34°C, 37°C, and 39.5°C, to assess the target specificity of secondary CTL.

As illustrated in Figure 8, resting memory anti-PV CTL were restimulated to a similar extent by peritoneal exudate cells infected with either ts 488 or wt PV. At the temperature permissive for the mutant (34°C), ts 488-infected PAK and 5R target cells were better targets for CTL generated by restimulation with wt and ts 488. At 37°C, the difference becomes less significant. At 39.5°C, the difference remains insignificant for cultures restimulated with ts 488, and is reversed for cultures restimulated with wt PV, where wt-infected targets were lysed slightly better.

FIGURE 8: COMPARISON OF WT AND TS 488 PV AS STIMULATORS AND AS TARGETS IN A SECONDARY ANTI-PV CYTOTOXIC RESPONSE

Five weeks after immunizing mice with wt PV, spleen cells were restimulated *in vitro* at 37°C for five days with antigen presenting cells infected with either wt (A) or ts488 (B) PV. Subsequently, cells were harvested for a ^{51}Cr release assay with PAK and 5R targets uninfected (—), or infected with wt (•••) or ts 488 PV (▬▬▬) at 34, 37, and 39.5°C. The ratio of effector lymphocytes to target cells was 40:1. The duration of the assay was 7.5 hours, and spontaneous release was generally less than 25%.


A
100
80
60
40
20
PER CENT SPECIFIC ^{51}CHROMIUM RELEASE
PAK
5R
34°C
37°C
39.5°C

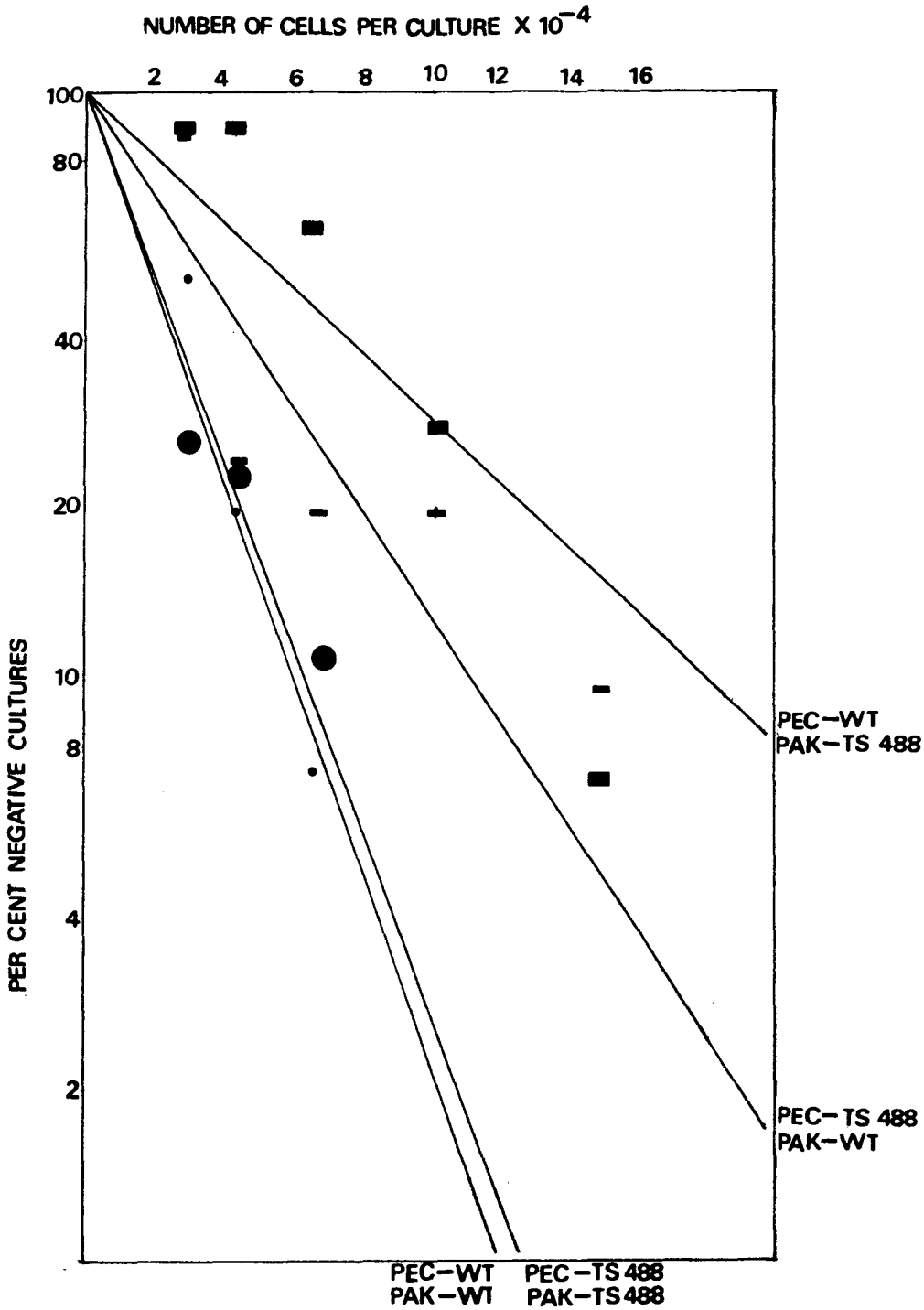

B
100
80
60
40
20
PER CENT SPECIFIC ^{51}CHROMIUM RELEASE
PAK
5R
34°C
37°C
39.5°C

C/2.2.3. Limiting Dilution Analysis for Wt and Ts 488 Restricted CTL Precursor Frequency

Since a comparable level of killing of target cells infected with either ts 488 or wt PV was observed, the CTL precursor (CTLp) frequency was examined in a limiting dilution assay (LDA). These results are displayed in Figure 9. A relatively high CTLp frequency is observed for wt-primed spleens restimulated with either ts 488 or wt PV, and assayed for cytolysis on PAK target cells infected with homologous virus. Consistent with the earlier results displayed in Figure 1, lymphocytes restimulated with wt PV and assayed on wt-infected PAK cells, the CTLp frequency was 1/28,011 with a 95% confidence interval of 1/18,249 to 1/42,993. For lymphocytes restimulated with ts 488 and assayed for cytolysis on ts 488-infected PAK cells, the CTLp frequency was 1/27,849 with a 95% confidence interval of 1/18,526 to 1/41,865. The precursor frequency of anti-wt CTL generated by restimulation with ts 488 is approximately half that generated by restimulating with homologous wt: 1/57,420 with a 95% confidence interval of 1/39,372 to 1/83,742. Only a small number of CTL that are restimulated with wt PV lyse ts 488-infected target cells. The frequency of these CTLp is 1/119,301 with a 95% confidence interval of 1/77,798 to 1/182,944.

FIGURE 9: LIMITING DILUTION ASSAY FOR WT AND TS 488 PV CTL PRECURSOR FREQUENCY

Five weeks after inoculating mice with wt PV, spleen cells were cocultured at limiting dilution with feeder cells and stimulator peritoneal exudate cells (PEC) infected with either wt or ts 488 PV. Cultures were incubated for five days at 37°C and then cytotoxicity was assayed in a ^{51}Cr release with PAK targets infected with either wt or ts488 PV. CTLp frequencies were: PEC-wt, PAK-wt (• •) 1/28,011 with 95% confidence interval from 1/18,249 to 1/42,993 and x^2 of 0.98; PEC-wt, PAK-ts 488 (■ ■) 1/119,301 with 95% confidence interval from 1/77,798 to 1/182,944 and x^2 of 8.3; PEC-ts 488, PAK-ts 488 (●●) 1/27,849 with 95% confidence interval from 1/18,526 to 1/41,865 and x^2 of 2.2; and PEC-ts 488, PAK-wt (▬ ▬) 1/57,420 with 95% confidence interval from 1/39,372 to 1/83,742 and x^2 of 9.7. As described earlier, spontaneous release was determined by incubating target cells with stimulator and feeder cells alone. 24 replicate wells were used in the determination of each point. Wells were scored as positive if the counts released from a well were greater than or equal to 3 SD above spontaneous release.


NUMBER OF CELLS PER CULTURE X 10^{-4}
2
4
6
8
10
12
14
16
100
80
40
20
10
8
4
2
PER CENT NEGATIVE CULTURES
PEC–WT
PAK–TS 488
PEC–TS 488
PAK–WT
PEC–WT
PAK–WT
PEC–TS 488
PAK–TS 488

Where bulk killing assays at 37°C indicated comparable levels of lysis after restimulation with either ts 488 or wt PV, limiting dilution assays indicated greater discrepancies in CTL precursor frequencies. That secondary restimulation of wt-primed spleen cells with ts 488 corresponds to a decreased frequency of anti-wt PV CTL precursors suggests that ts 488 and wt PV are not completely cross reactive at the level of antigen presentation. Similarly, that secondary restimulation with wt PV corresponds to different frequencies of anti-wt and anti-ts 488 CTL suggests that ts 488 and wt PV are not completely cross reactive at the level of CTL target recognition.

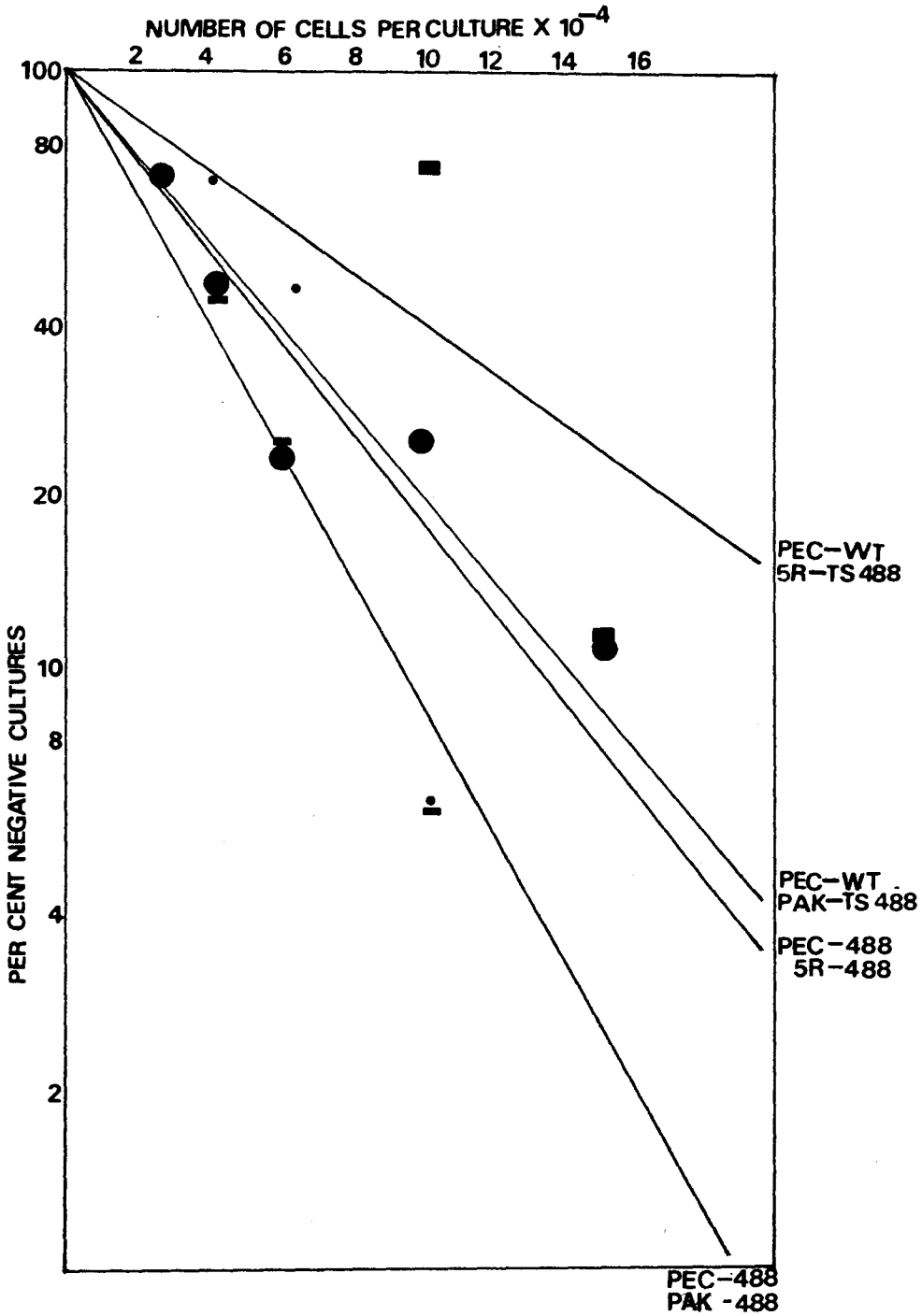
A final limiting dilution assay was used to examine whether wt-primed spleens restimulated with either ts 488 or wt PV showed different abilities to recognize ts 488 as a result of variable association with H-2K^b. Variable abilities to recognize virus in the context of H-2K^b were observed. These results are illustrated in Figure 10. The greatest CTLp frequency was observed by spleen cells restimulated with ts 488 and assayed for cytolysis against syngeneic PAK targets infected with homologous virus: 1/36,441 with a 95% confidence interval from 1/23,783 to 1/55,835. Slightly fewer CTLp are stimulated by ts 488 against K^b-compatible 5R targets infected with homologous virus: 1/48,044 with a

95% confidence interval from 1/31,351 to 1/73,623. These results suggest that approximately three-quarters of the population of anti-ts 488 CTL are restricted by the H-2K^b molecule. Spleen cells restimulated with wt PV stimulated few CTLp to lyse syngeneic PAK targets infected with ts 488: 1/49,995 with a 95% confidence interval from 1/32,602 to 1/76,666. Furthermore, consistent with the previous assay, restimulation with wt PV poorly stimulated CTLp against the K^b-compatible 5R target infected with ts 488: 1/156,844 with a 95% confidence interval from 1/85,899 to 1/286,384.

These results indicate that ts 488 may restimulate a greater number of CTLp to recognize virus in combination with K^b than wt PV does. This level of genetic restriction could be related to the observed superiority of ts 488 compared to wt PV in assays using 5R cells as targets in cytolysis assays. Additionally, at the level of restimulation, ts 488 generates more anti-wt PV CTL than vice versa. Taken together, this data indicates that CTL generation and activity may be affected by genetic restriction to either the K or D end of the H-2 complex.

FIGURE 10: LIMITING DILUTION ASSAY COMPARING K^B-RESTRICTED CTL PRECURSOR FREQUENCY OF SECONDARY CULTURES RESTIMULATED WITH TS 488 OR WT PV

Five weeks after immunizing mice with wt PV, spleen cells were cocultured at limiting dilution with feeder cells and peritoneal exudate cells (PEC) infected with either wt or ts 488. Cultures were incubated for five days and then assayed for cytotoxicity at 37°C in a ^{51}Cr release assay with ts 488-infected syngeneic PAK targets, or K^b-compatible 5R targets. CTL precursor frequencies were calculated by the Maximum Likelihood Method of Porter and Berry (1964): PEC-wt, PAK-ts 488 (●) 1/49,995 with a 95% confidence interval from 1/32,602 to 1/76,666 and x^2 of 9.1; PEC-wt, 5R-ts 488 (■) 1/156,844 with 95% confidence interval from 1/85,899 to 1/286,384 and x^2 of 10.0; PEC-ts 488, PAK-ts 488 (▬) 1/36,441 with 95% confidence interval from 1/23,783 to 1/55,835 and x^2 of 5.8; PEC-ts 488, 5R-ts 488 (•) 1/48,044 with 95% confidence interval from 1/31,351 to 1/73,623 and x^2 of 7.4. Spontaneous release was determined by incubating target cells with stimulator and feeder cells alone. 24 replicate wells were used in the determination of each point. Wells were scored as positive if the counts released exceeded that from control wells by at least 3 SD.


NUMBER OF CELLS PER CULTURE X 10^{-4}
2
4
6
8
10
12
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16
100
80
40
20
10
8
4
2
PER CENT NEGATIVE CULTURES
PEC–WT
5R–TS 488
PEC–WT
PAK–TS 488
PEC–488
5R–488
PEC–488
PAK -488

D./ DISCUSSION

D/1. SPECIFICITY OF THE ANTI-PV CELLULAR RESPONSE

These studies closely examined the specificity of the cell-mediated immune response to PV. A population of H-2K^b-restricted anti-PV CTL was detected by cytolysis assays, and limiting dilution assays. Subsequently, the viral target specificity of anti-PV CTL was studied with a panel of temperature sensitive (ts) mutants of PV. These experiments attempted to identify the predominant target antigen by correlating specific cytolysis with protein expression. One mutant virus, ts 488, appeared to be superior to wt PV as a target. The superior lysis was predominant in 5R target cells, which were compatible at K^b. Consistent with this, limiting dilution assays indicated that three-quarters of anti-ts 488 CTL precursors were restricted by K^b. Additionally, antigen presenting cells infected with ts 488 appeared to be superior to those infected with wt PV as a restimulator in secondary *in vitro* cultures of wt-primed CTL. The information from these studies of the cellular response to ts 488 or wt PV combined with self H-2 molecules may ultimately facilitate the generation of anti-PV CTL clones, and studies of cytotoxic T cell specificity.

D/1.1. H-2 Specificity of Anti-PV CTL

Studies of anti-viral immune responses have shown that CTL may be regulated by either K or D class I antigens of the H-2 complex (Zinkernagel et al, 1985). The D locus encodes at least four distinct gene products: D, L, M, and R (Ciavarra and Forman, 1982). The role of either the K or D region in restricting anti-viral CTL in a particular haplotype is usually elucidated in assays of cytolysis between appropriate haplotypes of effectors and target cells. In addition, inhibition of cytolysis with monoclonal antibodies to private epitopes of K or D molecules can reveal the molecules which regulate CTL function (O'Neill, 1986).

In this study, the results of cytolysis by primary effectors showed that CTL from PV-primed C57Bl/6 mice appeared to lyse infected targets *in vitro* in an H-2K^b-dependent manner. The Bl0.A(2R) effectors, failed to exhibit a high level of lysis, even against D^b-compatible PAK targets. However, K^b-end homology resulted in lysis comparable to that between syngeneic C57Bl/6 effectors and PAK target cells. Thus, it appears that the primary anti-PV cytotoxic response is predominantly regulated by the K^b locus of H-2.

Studies to determine the frequency of end-restricted CTL precursors (CTLp) failed to reveal the same preponderance of K^b-end restricted killers that had

been observed in primary bulk spleen lymphocytes. Approximately half the total number of anti-PV CTL precursors were regulated by the K^b end. This outcome may have been due to the secondary *in vitro* restimulation in contrast to the K^b restriction observed in primary effector population. Alternatively, the discrepancy between the end restriction observed in bulk and limiting dilution cultures may have resulted from a number of other variables (Lefkovits and Waldmann, 1984). The limiting dilution assay does not distinguish between clone sizes; conversely the bulk assay may reveal variable levels of lysis as a result of variable clone sizes. Possibly the cellular interactions upon which development or burst size of K^b-restricted CTL depends, may be limited in limiting dilution cultures. Perhaps in limiting dilution culture, the proportion of suppressor to helper cells and factors which interact with the K-restricted CTL increases, thus preventing full expression of K-restricted cytotoxicity.

Restriction of CTL responses to one or the other H-2 end locus has been observed in a number of systems other than the anti-PV response studied here. In bulk killing assays, cloned CTL lines specific for vesicular stomatitis virus (VSV) have been observed to recognize virus restricted predominantly by the D locus in the H-2^d system (Rosenthal et al, 1983). With specific

monoclonal antibodies, the function of anti-VSV CTL has been linked to the L^d molecule (Ciavarra and Forman, 1982). In the case of influenza, serological studies of the H-2D restricted CTL response have identified H-2L in restricting the cell mediated response (Blanden et al, 1979). As described earlier, the response to LCMV appears to be regulated by D-end determinants as indicated by the strain-dependent immunopathologic outcome (Zinkernagel et al, 1985a,b). In contrast to LCMV, the data from this study indicated that anti-PV lysis in primary bulk culture was predominantly regulated by K^b. Further, approximately one half of secondary anti-PV CTL were restricted by antigen in addition to molecules encoded by the K end of the $H\text{-}2^b$ complex.

D/1.2. Viral Specificity of Anti-PV CTL

Studies of anti-viral CTL specificity have become increasingly important in the pursuit of vaccines. A number of approaches in elucidating viral specificity have been developed. Temperature sensitive mutants and reassortant viruses allow correlation of protein expression with the ability of the virus to serve as a target (Zinkernagel and Rosenthal, 1981). Liposomes containing whole or fragmented molecules within the same

membrane bilayer reveal target requirements of CTL (Weinberger et al, 1985). Molecular techniques of DNA mediated gene transfer, viral recombinants and synthetic peptides allow even finer dissection of anti-viral CTL specificity (Zinkernagel and Rosenthal, 1981).

D/1.2.1. Temperature Sensitive Mutants to Identify the PV Target

A panel of temperature sensitive mutants of wild type PV were examined with the hope of elucidating the finer virus specificity of anti-PV CTL generated upon primary *in vivo* immunizations. Mutant viruses had been characterized in five groups depending on the production of nucleoprotein and glycoprotein precursor as measured by polyacrylamide gel electrophoresis, and the expression of surface glycoprotein as measured by indirect immunofluorescence. It was hoped that the ability of these mutants to serve as targets for cytolysis would indicate which element may be most important.

TABLE 3: Summary of Relative Lysis of 5R Targets Infected with ts Mutants, and their Protein Phenotypes

ts mutant (group)	34°C Lysis	39.5°C Lysis	39.5°C GPC[a]	39.5°C NP[b]	39.5°C IIF[c]
488 (A)	+++	++/+++	normal	normal	normal
5 (B)	+++	++	reduced	absent	absent
13 (C)	++	++	normal	increased	absent
908 (C)	+++	-	normal	increased	absent
2.2 (D)	+	-	reduced	reduced	reduced
11 (E)	+	+	reduced	reduced	reduced

a Nucleoprotein detected in polyacrylamide gel electrophoresis (PAGE).

b Glycoprotein precursor detected in PAGE.

c Surface molecules detected by indirect immunofluorescence.

The summary of results displayed in Table 3 shows that there is no clear correlation between expression of nucleoprotein or glycoprotein and lysis. All of the ts mutants, but those in group E, displayed normal wt protein phenotypes at 34°C, and the deficient phenotypes described in Table 3 at 39.5°C. Lysis does not appear to correlate with expression of GPC or NP, since inhibition of lysis was observed with normal or excessive levels of either one. Lysis appears to correlate loosely with the level of surface protein detected by indirect immunofluorescence, where decreases of its levels correspond to inhibited lysis. Ts 908 exhibited a significant decrease in its ability to form targets for lysis at 39°C, corresponding to a lack of surface protein as detected by indirect immunofluorescence. This information suggests that the expression of surface glycoprotein(s) GP1 and/or GP2 may be involved in CTL target formation. It is possible that proteins which are serologically detected may lack the epitopes important in cell-mediated immunity, and vice versa, thus precluding clear conclusions from being drawn. Other approaches may be required to clearly elucidate the viral specificity of anti-PV CTL.

Studies of increasing numbers of viral systems indicate that internal or nonstructural components of viruses may be involved in target structures, in

contrast to predictions of surface moieties. Evidence obtained from LCMV reassortant viruses which were made between the strains Armstrong and Pasteur showed that CTL from both primary immunized spleens as well as long-term culture recognized gene products encoded by the S RNA segment, and not the L RNA (Riviere et al, 1986). This suggests that either the NP or GPC of LCMV is the important target molecule. The nucleoprotein has been identified as a major target structure in anti-viral immunity to vesicular stomatitis virus (Puddington et al, 1986), and respiratory syncitial virus (Bangham et al, 1986).

Studies of influenza support the importance of internal viral molecules as CTL targets. The unglycosylated, non-transmembrane nucleoprotein has been identified as a target for the majority of cross-reactive CTL generated in the anti-influenza cell-mediated immune response. The specific epitopes which serve as targets have been defined by fragments of the molecule (Townsend et al, 1985), and more recently by short synthetic peptides (Townsend et al, 1986). These peptides of less than 15 amino acids represent the most conserved regions of the influenza nucleoprotein. They sensitized the cellular immune response better than the entire protein, thus supporting observations that antigens are degraded and then displayed to class I

restricted CTL (Bastin et al, 1987). That intracellular viral proteins may be degraded and displayed is further supported by experiments where an internal molecule was created from a surface molecule of influenza. The haemagglutinin molecule is the predominant transmembrane protein of the virus. Deleting the N-terminal signal peptide from cDNA encoding the haemagglutinin produced a short-lived, unglycosylated, intracellular protein. Nevertheless, CTL primed by the complete molecule could still see the mutant intracellular haemagglutinin as a target (Townsend, 1986). Consistent with the hypothesis of intracellular proteins serving as target structures, nucleoprotein has been demonstrated on the surface of LCMV-infected cells by immunoprecipitation with a monoclonal antibody prior to polyacrylamide gel electrophoresis (Zeller et al, 1986). This information taken together indicates that the relevant epitopes of intracellular proteins may be processed and displayed as important CTL targets.

Contrasting studies show the importance of surface molecules as CTL targets. Such observations have been made with haemagglutinin as a target for anti-influenza CTL (Bennink et al, 1986), and with glycoproteins gD (Zarling, 1986) and gC (Rosenthal et al, 1987) as targets for anti-herpes simplex virus CTL.

Preliminary evidence from anti-PV CTL suggests that

surface moieties detected by indirect immunofluorescence correlate with appropriate CTL target structures. A more specific approach with vaccinia recombinants containing cloned genetic material from PV is underway. Identification of the target molecules for anti-PV CTL may prove useful in generating CTL clones.

D/1.2.2. Ts 488 as a Target and as a Restimulator

Ts 488 had the same phenotype as wild type PV, as assessed by indirect immunofluorescence and by polyacrylamide gel electrophoresis (Prakash, Master's thesis, 1985). Surface and intracellular viral components were observed at both permissive and non-permissive temperatures; however, replication was attenuated at the non-permissive temperature as observed by comparisons of virus titres at both temperatures. Ts 488 was studied more closely because of its apparent superiority as a target and restimulator of the anti-PV response at permissive and non-permissive temperatures.

Examination of lytic specificity of primary anti-PV cytotoxic cells suggested that 5R cells infected with ts 488 appeared to be superior targets compared to those infected with wt PV, at both permissive and at physiologic temperatures. At the non-permissive temperature, infection with wt PV appeared to generate

better targets for lysis. That the non-permissive temperature mitigates the ability of ts 488 to be a target suggests that the virus titre determines the number of relevant antigenic proteins formed. The ability of ts 488 to be a superior viral target than wt PV in 5R targets suggests that the cell line may allow antigenic determinants of the former virus to be better processed in 5R targets, or to associate that much better with the K^b-end locus. Alternatively, other loci in PAK cells may interfere via steric hindrance or more direct inhibition, with the ability of wt virus to be processed and to associate with the necessary self molecules. Rising levels of lysis of 5R target cells infected with wt PV suggest that increasing temperature augmented the role of K^b in regulating the response to wt PV.

For antigen presenting cells infected with ts 488 to stimulate proliferation of wt-primed CTL comparable to those infected with the wild type strain suggests that the ts 488 antigens were well processed and presented. It may take longer for the antigens of ts 488 to be processed and presented in combination with self molecules, compared to wt PV, as supported by the 24 hour delay in the peak of proliferation in response to ts 488.

For secondary restimulations <u>in vitro</u>,

macrophages infected with the ts 488 appear to elicit CTL responses comparable to macrophages infected with wt PV, despite primary immunization with the wt virus. It appears that macrophages infected with the mutant virus may elicit potent antigens, hence potent lytic effectors.

As a target, the superiority of ts 488 observed particularly at the permissive temperature for the mutant in the K^b-end compatible 5R cell line, again reinforces the same interpretations as described in the primary CTL response: that ts 488 may be better processed and displayed by K^b-compatible 5R cells.

Attenuation of the mutant virus titre by at least 100-fold in the non-permissive assay does not prevent formation of the immunogenic antigens in the anti-ts 488 response. Furthermore, even CTL generated against the wt virus recognize a significant number of target cells infected with the mutant virions. This indicates that the relevant antigens continue to be processed and displayed in sufficient number to elicit lysis.

Limiting dilution assays were undertaken to examine the effect of ts 488 introduced to wt-primed CTL precursors at the level of either the antigen presenting cell or the target cell. These results are schematically summarized in Figure 11. It appears that the epitopes involved in stimulating CTL are not

necessarily the same as those that form targets for cytolysis, as suggested by the superior ability of ts 488 to cross react with wt PV at the level of antigen presentation, compared with cross reactivity at the level of target cytolysis.

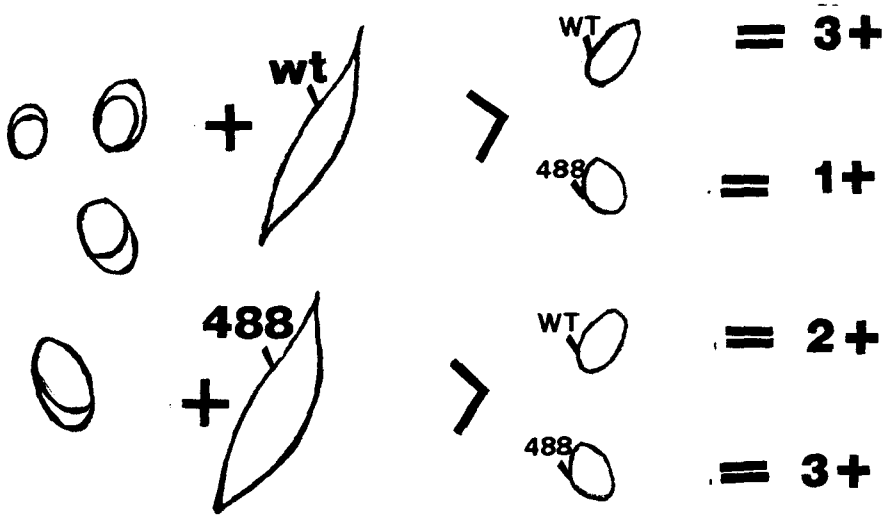
Restimulation of wt-primed memory CTL with ts 488 generated approximately half as many anti-wt CTL compared with restimulation by wt PV (Figure 9: CTLp frequencies of 1/28,011 and 1/57,420 respectively). Similarly, restimulation of wt-primed memory CTL with wt PV generated approximately one-fourth as many anti-ts 488 CTL compared with restimulation with homologous ts 488 (Figure 9: CTLp frequencies of 1/119,301 and 1/27,849 respectively). This data indicates that ts 488 and wt PV are not fully cross reactive at the level of antigen presentation despite comparable levels of lysis observed in bulk culture (Figure 8).

Consistent with the earlier observations of the superiority of ts 488 in 5R target cells, CTL precursor frequencies (Figure 10) indicate that the anti-ts 488 CTL response is more greatly regulated by the H-2K^b molecules than the anti-wt PV response: approximately one-half of the anti-wt CTL precursors are apparently regulated by H-2K^b, while approximately three-quarters of the anti-ts 488 CTL precursors are apparently regulated by H-2K^b.

FIGURE 11: SCHEMATIC REPRESENTATION OF WT AND TS 488 CROSS REACTIVITY IN LIMITING DILUTION

CTL precursor frequencies were assessed against PAK targets infected with virus as indicated five days after restimulation of wt-primed memory cells with virus-infected antigen presenting cells as indicated.

WT-PRIMED CTL MEMORY CELLS	+	VIRUS-INFECTED ANTIGEN PRESENTING CELLS	>	ASSAY ON VIRUS-INFECTED PAK TARGETS	=	LYTIC EFFECTORS



That a particular gene may influence the generation and activity of anti-viral CTL is supported by observations in the human system where HLA B37 was found to regulate CTL recognition of an epitope of influenza A nucleoprotein (McMichael et al, 1986). Similarly, studies with LCMV suggested that the fine specificity of LCMV-specific CTL is a function of the H-2 region. More specifically, cross-reactivity between different strains of LCMV varies in different strains of mice. These results indicated that target viral epitopes may be different in the context of various H-2 restricting elements (Ahmed et al, 1984). In this way, cross reactivity between wt and ts mutants of PV may be observed to differ among mice of different haplotypes.

D/1.2.3. <u>The Model and its Implications</u>

The studies described do not distinguish whether this CTL specificity is determined at the level of antigen presentation, the repertoire of helper T cells available, or the repertoire of CTL available for target cell recognition. Studies are beginning to clarify the fine specificity of the T-cell response. Using class II molecules as a model, new evidence indicates that antigens directly bind class II MHC molecules, thus forming a trimolecular complex among H-2, antigenic peptide, and T-cell receptor (Guillet et al, 1987). It was found that protein derived peptides bind directly to

Ia molecules, with dominant binding to the restriction element (Buus et al, 1987). This binding is very slow, and very stable (Marx, 1987) with a half life potentially as great as 30 hours (Marrack, 1987). The peptides bind to Ia molecules with which they share a great degree of homology in the binding site, as determined by amino acid sequencing (Guillet et al, 1987). Upon binding between MHC and the corresponding peptide epitope, termed the internal agretope or motif, the peptide may undergo conformational changes to expose an internal epitope to which T cells may bind and respond (Schwartz, 1987; Guillet et al, 1987). Peptides restricted by the same Ia molecule bind to that molecule via the same motif, as indicated by binding inhibition studies. Portions of both the α and β chains of Ia molecules compose this peptide binding site (Buus et al, 1987). The possibility exists for more than one motif to be associated with an MHC molecule (Guillet et al, 1987). These results suggest that quantitative differences in T-cell responsiveness may be determined by competition among peptides for direct binding to MHC molecules rather than competition for T-cell activation (Guillet et al, 1987).

The repertoire of T cells may preclude antigens from being restricted by the molecules to which they best bind. This is the case for a lambda phage CI

repressor peptide. The Class II molecule to which it binds best is not the restricting MHC molecule. In fact, the homology between the peptide and the Ia molecule to which it preferentially binds is so great that the immune system probably fails to distinguish the antigen from self. The T-cell clone specific for this and other apparently "self" molecules may have been deleted during ontogeny. Homology between the motif and the peptide appear to be required for binding, but near identity prevents the development of an immune response because of an apparent "hole" in the T cell repertoire. This indicates that MHC binding is necessary to elicit T cell activity, but not sufficient (Buus et al, 1987). Further studies are required to examine whether Class I molecules exhibit distinct motifs with which they bind antigenic peptides prior to T-cell binding.

The PV system under discussion may prove useful in confirming and extending this model of T cell recognition and specificity. Possibly the target epitopes of ts 488 bear greater homology with the K^b molecule than those of wt PV, and thus exhibit greater binding to the restricting molecule, and increased regulation by the molecule. Despite apparent decreases in CTL precursor frequency with the introduction of ts 488, similar levels of lysis were observed in bulk culture with the predominantly K^b-restricted mutant.

This system may prove useful in elucidating the more precise role of host "self" molecules in cell mediated immunity. In addition, this system may allow comparison of recognition of antigen plus "self" in secondary restimulation with that of target recognition.

To further study the fine specificity of the anti-PV response, new molecular approaches might be undertaken. Murine L cells transfected with either influenza nucleoprotein or haemagglutinin indicated that the former molecule is very important in the CTL response (Townsend et al, 1984). Cloning genes for the nucleoprotein and the glycoprotein allow for separation of potential target molecules. Insertion of the cDNA into vectors such as vaccinia would enable analysis of these moities with respect to immunogenicity and target specificity. Such conclusive studies are exemplified by those using vaccinia virus recombinants containing influenza surface haemagglutinin which can prime and stimulate the CTL response (Bennink et al, 1984). The epitopes of PV that are involved in the cell-mediated immune response may be more precisely identified by using fragments of the molecules, and by synthetic peptides and appropriate deletion mutants. This information may be useful in generating cloned lines of anti-PV CTL. Such clones would allow studies of the CTL fine target specificity, of their mechanism of action,

and of their role *in vivo*.

D/2. SUMMARY AND CONCLUSIONS

These studies have focussed on the genetic and viral specificity of the anti-PV cell mediated immune response. The response appears to be regulated in part by the K locus of the H-2^b complex in C57Bl/6 mice, as determined by cytolysis assays, and limiting dilution assays.

Examination of the target specificity of the primary CTL effector using a panel of temperature sensitive mutants of PV gave equivocal results, with no clear indication of whether the nucleoprotein or the glycoprotein was most important. The results suggest that surface protein detected by indirect immunofluorescence may be important in the formation of CTL targets.

Finally, a mutant virus with a normal phenotype and an attenuated titre at non-permissive temperatures, was observed to be a potent stimulator of anti-PV immunity as well as a good target. The ability of this mutant, ts 488, to act as a target was better than wt PV in the K^b-end compatible 5R target than in the syngeneic PAK target. Restimulation of wt-primed memory CTL with ts 488 produced a level of lysis similar to restimulation with wt PV. Limiting dilution assays suggested the

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anti-ts 488 response was more greatly restricted by K^b, a feature which may be involved in its superior ability as a stimulator and as a target in the anti-PV response.

This system could provide an opportunity to examine the association and recognition of viral antigen plus "self" at the level of antigen presentation and target cytolysis because of the observed augmented role of K^b in the anti-ts 488 response compared with the anti-wt PV response. In all likelihood, this mutant would not facilitate the generation of anti-wt PV CTL clones because of the apparent lack of cross reactivity between wt and ts 488.

APPENDIX A

Using the Maximum Likelihood Method of Porter and Berry (1964) to calculate CTL precursor frequency, the results of five dilutions were transformed by log functions to create a linear relationship of the data where the distributions of errors was nearly normal. To calculate the maximum likelihood, iterative calculations estimating the log of the number of intact CTL precursors per well were applied at each dilution until the error was negligible, so the difference between successive iterations of the estimate of the log was negligible, and the solution was closely approached. The statistical value of the 95% confidence interval was calculated based on the solution. The x^2 value, as a measure of inconsistency of the data, is based on the degrees of freedom, thus indicating how well the observed numbers compare with the expected numbers. This statistic is a measure of the reliability of the solution.

E./ REFERENCES

Ahmed R., J.A. Byrne, M.B.A. Oldstone. 1984. Virus Specificity of Cytotoxic T Lymphocytes Generated During Acute Lymphocytic Choriomeningitis Virus Infection: Role of the H-2 Region in Determing Cross-Reactivity for Different Lymphocytic Choriomeningitis Virus Strains. J. Virol., 51: 34.

Askonas B.A., A. Mullbacher, R.B. Ashman. 1982. Cytotoxic T-Memory Cells in Virus Infection and the Specificity of Helper T Cells. Immunol., 45: 79.

Auperin D.A., V. Romanowski, M. Galinski, D.H.L. Bishop. 1984. Sequencing Studies of Pichinde Arenavirus S RNA Indicate a Novel Coding Strategy, an Ambisense Viral S RNA. J. Virol., 52: 897.

Banerjee S.N., M.B. Buchmeier, W.E. Rawls. 1975,6. Requirement of Cell Nucleus for the Replication of an Arenavirus. Intervirol., 6: 190.

Bangham C.R.M., D.J. Openshaw, L.A. Ball, A.M.Q. King, G.W. Wertz, B.A. Askonas. 1986. Human and Murine Cytotoxic T Cells Specific to Respiratory Syncitial Virus Recognize the Viral Nucleoprotein (N), but not the major Glycoprotein (Gl) Expressed by Vaccinia Virus Recombinants. J. Immunol., 137: 3973.

Bastin J., J. Rothbard, J. Davey, I. Jones, A. Townsend. 1987. Use of Synthetic Peptides of Influenza Nucleoprotein to Define Epitopes Recognized by Class I-Restricted Cytotoxic T Lymphocytes. J. Exp. Med., 165: 1508.

Bennink J.R., J.W. Yewdell, G.L. Smith, B. Moss. 1986. Recognition of Cloned Influenza Virus Haemagglutinin Products by Cytotoxic T Lymphocytes. J. Virol., 57: 786.

Bennink J.R., J.W. Yewdell, G.L. Smith, G. Moller, B. Moss. 1984. Recombinant Vaccina Virus Primes and Stimulates Influenza Haemagglutinin-Specific Cytotoxic T Cells. Nature, 311: 578.

Bevan M.J. 1987. Antigen Recognition. Class Discrimination in the World of Immunology. Nature, 325: 192.

Blanden R.V., A. Mullbacher, R.B. Ashman. 1979. Different D End-Dependent Antigenic Determinants are Recognized by H-2-Restricted Cytotoxic T Cells Specific for Influenza and Bebaru Viruses. J. Exp. Med., 150: 166.

Brooks C.G. 1983. Reversible Induction of Natural Killer Cell Activity in Cloned Murine Cytotoxic T Lymphocytes. Nature, 305: 155.

Brunet J.F., M. Dosseto, F. Denizot, M-G. Mattei, W.R. Clark, T.M. Haqqi, P. Ferrier, M. Nabholz, A-M. Schmitt-Verhulst, M.F. Luciani, P. Golstein. 1986. The Inducible Cytotoxic T-Lymphocyte-Associated Gene Transcript CTLA-1 Sequence and Gene Localization to Mouse Chromosome 14. Nature, 322: 268.

Buchmeier M.J., S.R. Gee, W.E. Rawls. 1977. Antigens of Pichinde Virus: I. Relationship of Soluble Antigens Derived from Infected BHK-21 Cells to the Structural Components of the Virion. J. Virol., 22: 175.

Buchmeier M.J., W.E. Rawls. 1977. Variation between strains of hamsters in the Lethality of Pichinde Virus Infections. Infect. Immun., 16: 413.

Buus S., A Sette, S.M. Colon, C. Miles, H.M. Grey. 1987. The Relation Between Major Histocompatibility Complex (MHC) Restriciton and the Capacity of Ia to Bind Immunogenic Peptides. Science, 235: 1353.

Byrne J.A., R. Ahmed, M.B.A. Oldstone. 1984. Biology of Cloned Cytotoxic T Lymphocytes Specific for Lymphocytic Choriomeningitis Virus. I: Generation and Recognition of Virus Strains and H-2b Mutants. J. Immunol., 133: 433.

Cantrell D.A., K.A. Smith. 1984. The Interleukin-2 T-Cell System: A New Cell Growth Model. Science, 224: 1312.

Carter M.F., N. Bisival, W.E. Rawls. 1974. Polymerase Activity of Pichinde Virus. J. Virol., 13: 577.

Carter M.F., N. Biswal, W.E. Rawls. 1973. Characterization of Nucleic Acid of Pichinde Virus. J. Virol., 11: 61.

Chen L-K., B. Tourbieille, G.F. Burns, F.H. Bach, D. Mathieu-Mahul, M. Sasportes, A. Bensussan. 1986. Interferon: a Cytotoxic T Lymphocyte Differentiation Signal. Eur. J. Immunol., 16 767.

Chen L-K., D. Mathieu-Mahul, F.H. Bach, J. Dausset, A. Bensussan, M. Sasportes. 1986. Recombinant Interferon gamma can induce Rearragement of T-Cell Antigen Receptor gamma-chain genes and Maturation to Cytotoxicity in T-Lymphocyte Clones in vitro. PNAS (USA), 83: 4887.

Ciavarra R., J. Forman. 1982. H-2L Restricted Recognition of Viral Antigens. In the H-2^d Haplotype, anti-vesicular stomatitis virus Cytotoxic T Cells are Restricted Solely by H-2L. J. Exp. Med., 156: 778.

Cohen J.J. 1986. Mechanisms of Immune Function: Cell Mediated Reactions. Prog. Clin. Biol. Res., 213: 445.

Dembic Z., H. von Boehmer, M. Steinmetz. 1986. The Role of T-Cell Receptor alpha and beta genes in MHC-Restricted antigen Recognition. Immunol. Today, 7(10): 308.

Dimock K., D.G. Harnish, G. Sisson, W-C. Leung, W.E. Rawls. 1982. Synthesis of Virus-Specific Polypeptides and Genomic RNA during the Replicative Cycle of Pichinde Virus. J. Virol., 43: 273.

Djeu J.Y., J.A. Heinbaugh, H.T. Holden, R.B. Herberman. 1979. Augmentation of Mouse Natural Killer Cell Activity by Interferon and Interferon Inducers. J. Immunol., 93: 175.

Dunlop M.B.C., P.C. Doherty, R.M. Zinkernagel, R.V. Blanden. 1976. Secondary Cytotoxic Cell Response to Lymphocytic Choriomeningitis Virus. II) Nature and Specificity of Effector Cells. Immunol., 31: 181.

Dunlop M.B.C., R.V. Blanden. 1976. Secondary Cytotoxic Cell Response to Lymphocytic Choriomeningitis Virus. I) Kinetics of Induction in vitro and yields of effector cells. Immunol., 31: 171.

Dutko F.J., E.A. Wright, C.J. Pfau. 1976. The RNA's of Defective Interfering Particles of Pichinde Virus. J. Gen. Virol., 31: 417.

Eisen H.N., M.A. Liu, D.M. Kranz. 1985. The Central Role of the Antigen-Specific Receptor Complex in Triggering Cytolytic Activity of Cytotoxic T Lymphocytes. Biosci. Rep., 15: 885.

Epplen J.T., F. Bartels, A. Becker, G. Nerz, M. Prester, A. Rinaldy, M.M. Simon. 1986. Change in Antigen-Specificity of Cytotoxic T Lymphocytes is Associated with the Rearrangement and Expression of a T-Cell Receptor beta-chain Gene. PNAS (USA), 83: 4441.

Farber F.E., W.E. Rawls. 1975. Isolation of Ribosome-Like Structures from Pichinde Virus. J. Gen. Virol., 26: 21.

Fleischer B. 1986. Lysis of Bystander Target Cells After Triggering of Human Cytotoxic T Lymphocytes. Eur. J. Immunol., 16: 1021.

Fleischer B., H. Schrezenmeier, H. Wagner. 1986. Function of the CD4 and CD8 Molecules on Human Cytotoxic T Lymphocytes: Regulation of T-Cell Triggering. J. Immunol., 136: 1625.

Germain R.N. 1986. The ins and outs of Antigen Processing and Presentation. Nature, 322: 687.

Gershenfeld H.K., I.L. Weissman. 1986. Cloning of a cDNA for a T-Cell Specific Serine Protease from a Cytotoxic T Lymphocyte. Science, 232: 854.

Gillis S., K.A. Smith. 1977. Long Term Culture of Tumour Specific Cytotoxic T Cells. Nature, 268: 154.

Gillis S., M.M. Ferm, W. Ou, K.A. Smith. 1978. T-Cell Growth Factor: Parameters of Production and Quantitative Microassay for Activity. J. Immunol., 120: 2027.

Goverman J., T. Hunkapiller, L. Hood. 1986. A Speculative View of the Multicomponent Nature of T-Cell Antigen Recognition. Cell., 45: 475.

Gray L.S., J.H. Russell. 1986. Cytolytic T Lymphocyte Effector Function Requires Plasma Membrane Chloride Flux. J. Immunol., 136: 3032.

Green L.M., J.L. Reade, D.F. Ware, P.E. Devlin, C-M. Liang, J.J. Devlin. 1986. Cytotoxic Lymphokines Produced by Cloned Human Cytotoxic T Lymphocytes. II) A Novel CTL-Produced Cytotoxin that is Antigenically Distinct from TNF and alpha Lymphotoxin. J. Immunol., 137: 3488.

Green L.M., M.L. Stern, D.L. Haviland, B.J. Mills, D.F. Ware. 1985. Cytotoxic Lymphokines Produced by Cloned Human Cytotoxic T Lymphocytes. I) Cytotoxins Produced by Antigen-Specific and NK-Like CTL are Dissimilar to Classical Lymphotoxins. J. Immunol., 135: 4034.

Guillet J-G., M-Z. Lai, T.J. Briner, S. Buus, A. Sette, H.M. Grey, J.A. Smith, M.L. Gefter. 1987. Immunological Self, Nonself Discrimination. Science, 235: 865.

Haars R., M. Kronenberg, W.M. Gallatin, I.L. Weissman. 1986. Rearrangement and Expression of T Cell Antigen Receptor Gamma Genes During Thymic Development. J. Exp. Med., 164: 1.

Hackett J.Jr., M. Bennett, G.C. Koo, V. Kumar. 1986. Origin and Differentiation of Natual Killer Cells: III) Relationship Between the Precursors and Effectors of NK and NC Activity. Immunol Res., 5: 16.

Hardt C., T. Diamanstein, H. Wagner. 1985. Signal Requirement for the in vitro differentiation of Cytotoxic T Lymphocytes (CTL): Distinct Soluble Mediators Promote Preactivation of CTL-Precursors, Clonal Growth and Differentiation into Cytotoxic Effector Cells. Eur. J. Immunol., 15: 472.

Harnish D.G., W-C. Leung, W.E. Rawls. 1981. Characterization of Polypeptides Immunoprecipitable from Pichinde Virus - Infected BHK-21 Cells. J. Virol., 38: 840.

Harnish D.G., K. Dimock, D.H.L. Bishop, W.E. Rawls. 1983. Gene Mapping in Pichinde Virus: Assignment of Viral Polypeptides to Genomic L and S RNA's. J. Virol., 46: 638.

Henney C.S., K. Kuribayashi, D.E. Kern, S. Gillis. 1981. Interleukin 2 Augments Natural Killer Cell Activity. Nature., 291: 335.

Howard C.R., D.I.H. Simpson. 1980. The Biology of the Arenaviruses. J. Gen. Virol., 51: 1.

Johnson K.M., P.A. Webb, G. Justines. 1973. Biology of Tacaribe Complex Viruses. In: Lehmann Grube F. (eds.) Lymphocytic Choriomeningitis and Other Arenaviruses. Springer; Berlin, Heidelberg, New York. pp. 241-258.

Kabelitz D., H. Kirchner, D. Armerding, H. Wagner. 1985. Recombinant Interleukin 2 Rapidly Augments Human Natural Killer Cell Activity. Cell Immunol., 93: 38.

Kaplan J., K. Wasserman. 1985. A Subpopulation of Allospecific Cytotoxic T-Cell Precursors with Phenotypic Characteristics of Natural Killer Cells. Nat. Immun. Cell Growth Regul., 4: 305.

Katz D.R., M. Feldmann, R. Tees, M.H. Schreier. 1986. Heterogeneity of Accessory Cells Interacting with T-Helper Clones. Immunology, 58: 167.

Kiessling R., E. Klein, H. Wigzell. 1975. "Natural" Killer Cells in the Mouse. I: Cytotoxic Cells with Specificity for Mouse Moloney Leukemia Cells. Specificity and Distribution According to Genotype. Eur. J. Imm., 5: 112.

Kim K.H., M.J. Solway, D.W. Thomas. 1985. Cellular Requirements for Antigen Processing by Antigen Presenting Cells: Evidence for Different Pathways in Forming the Same Antigenic Determinants. Cell. Immunol., 96: 267.

Klein J. 1975. Biology of the Mouse Histocompatibility-2 Complex. Principle of Immunogenetics Applied to a Single System. Springer-Verlag, New York.

Kouttab N.M., S. Mehta, J. Morgan, N. Tannir, C. Sahasrabuddhe, A.L. Maizel. 1984. Lymphokines and Monokines as Regulators of Human Lymphoproliferation. Clin. Chem., 30: 1539.

Lanier L.L., J.H. Phillips, H. Hackett Jr., M. Tutt, V. Kumar. 1986. Natural Killer Cells: Definition of a Cell Type Rather than a Function. J. Immunol., 137: 2735.

Lanzavecchia A. 1986. Is the T-Cell Receptor Involved in T-Cell Killing? Nature, 319: 778.

Lefkovits, I., H. Waldmann. 1984. Limiting Dilution Analysis of the cells of Immune System. I: The Clonal Basis of the Immune Response. Immunol. Today, 5: 265.

Lefrancois L., M.J. Bevan. 1985. Functional Modifications of Cytotoxic T-Lymphocyte T200 Glycoprotein Recognized by Monoclonal Antibodies. Nature, 314: 449.

Lefrancois L., L. Puddington, C.E. Machamer, M.J. Bevan. 1985. Acquisition of CTL-Specific Carbohydrate Differentiation Antigens. J. Exp. Med., 162: 1275.

Lehmann-Grube F. 1984. Portraits of Viruses-Arenaviruses. Intervirol., 22: 121.

Leiden J.M., J.D. Fraser, J.L. Strominger. 1986. The Complete Primary Structure of the T-Cell Receptor Genes from an Alloreactive Cytotoxic Human T-Lymphocyte Clone. Immunogenetics, 24: 17.

Leung W-C., M.F.K.L. Leung, W.E. Rawls. 1979. Distinctive RNA Transcriptase, Polyadenylic Acid Polymerase, and Polyuridylic Acid Polymerase Activities Associated with Pichinde Virus. J. Virol., 30: 98.

Leung W-C., W.E. Rawls. 1977. Virion Associated Ribosomes are not Required for the Replication of Pichinde Virus. Virol., 81: 174.

Lindahl K.F., H. Lemke. 1979. Inhibition of killer-target Cell Interaction by monoclonal anti-H-2 Antibodies. Eur. J. Immunol., 9: 526.

Lobe C.G., B.B. Finlay, W. Paranchych, V.H. Paetkau, R.C. Bleackley. 1986. Novel Serine Proteases Encoded by Two Cytotoxic T Lymphocyte Specific Genes. Science, 232: 858.

Lowenthal J.W., C. Tougne, H.R. MacDonald, K.A. Smith, M. Nabholz. 1985. Antigenic Stimulation Regulates the Expression of IL-2 Receptors on a Cytolytic T Lymphocyte Clone. J. Immunol., 134: 931.

Malek T.R., J.D. Ashwell. 1985. Interleukin 2 Upregulates Expression of its Receptor on a T Cell Clone. J. Exp. Med., 161: 1575.

Mannel D.N., W. Falk, W. Droge. 1983. Induction of Cytotoxic T Cell Function Requires Sequential Action of Three Different Lymphokines. J. Immunol., 130: 2508.

Marrack P. 1987. New Insights into Antigen Recognition. Science, 235: 1311.

Marx J.L. 1987. Histocompatibility Restriction Explained. Science, 235: 843.

Marx J.L. 1986. How Killer Cells Kill Their Targets. Science, 231: 1367.

McMichael A.J., F.M. Gotch, J. Rothbard. 1986. HLA B37 Determines an Influenza-A Virus Nucleoprotein Epitope Recognized by Cytotoxic T Lymphocytes. J. Exp. Med., 164: 1397.

Merluzzi V.J., M.D. Smith, K. Last-Barney. 1986. Similarities and Distinctions between Murine Natural Killer Cells and Lymphokine-Activated Killer Cells. Cell Immunol., 100: 563.

Merluzzi V.J. 1985. Comparison of Murine Lymphokine-Activated Killer Cells, Natural Killer Cells and Cytotoxic T Lumphocytes. Cell Immunol., 95: 95.

Mifune K., M. Carter, W.E. Rawls. 1971. Characterization Studies of the Pichinde Virus - A Member of the Arenavirus Group. Proc. Soc. Exp. Biol. Med., 136: 637.

Morgan D.A., F.W. Fuscetti, R. Gallo. 1976. Selective *in vitro* growth of T Lymphocytes from Normal Human Bone Marrows. Science, 193: 1007.

Murphy F.A., M.J. Buchmeier, W.E. Rawls. 1977. The Reticuloendothelium as the Target in a Virus Infection. Pichinde Virus Pathogenesis in Two Strains of Hamsters. Lab Invest., 37: 502.

Murphy F.A., S.G. Whitfield. 1975. Morphology and Morphogenesis of Arenaviruses. Bull. of WHO., 52: 409.

Nabholz M. 1983. Cytolytic T Lymphocytes. Ann. Rev. Immunol., 1: 273.

Neurath H., K.A. Walsh. 1976. Role of Proteolytic Enzymes in Biological Regulation. PNAS (USA), 73: 3825.

Oldstone M.B.A., R. Ahmed, J. Byrne, M.J. Buchmeier, Y. Riviere, P. Southern. 1985. Virus and Immune Responses: Lymphocytic Choriomeningitis Virus as a Prototype Model of Viral Pathogenesis. Brit. Med. Bul., 41: 70.

O'Neill H.C. 1986. Monoclonal Antibodies Specific for H-2K and H-2D Antigens on Cytotoxic T Cells can inhibit their Function. PNAS., 83: 1443.

Ortaldo J.R., L. Mason, R.B. Herberman. 1986. Relationship of Mouse Natural Killer Cells to Mouse Natural Cytotoxic Cells: Effector Cells and Possible Mechanism of Action. Immunol Res., 5: 25.

Pasternack M.S., H.N. Eisen. 1985. A Novel Serine Esterase Expressed by Cytotoxic T Lymphocytes. Nature, 314: 743.

Pedersen I.R. 1979. Structural Components and Replication of Arenaviruses. Adv. Virus Res., 24: 277.

Phillips J.H., L.L. Lanier. 1985. A Model for the Differentiation of Human Natural Killer Cells. Studies on the in vitro activation of leu-11+ Granular Lymphocytes with a Natural Killer-Sensitive tumor cell, K562. J. Exp. Med., 161: 1464.

Porter E.L.T., R.J. Berry. 1964. The Efficient Design of Transplantable Tumor Assays. Br. J. Cancer, 17: 583.

Prakash, M. 1986. M.Sc. Thesis. McMaster University.

Puddington L., M.J. Bevan, J.K. Rose, L. Lefrancois. 1986. N Protein is the Predominant Antigen Recognized by Vesicular Stomatitis Virus - Specific Cytotoxic T Cells. J. Virol., 60: 708.

Ramos B.A., R.J. Courtney, W.E. Rawls. 1972. Structural Proteins of Pichinde Virus. J. Virol., 10: 661.

Raulet D.H., M.J. Bevan. 1982. A Differentiation Factor Required for the Expression of Cytotoxic T-Cell Function. Nature, 296: 754.

Rawls W.E., M. Buchmeier. 1975. Arenaviruses: Purification and Physicochemical Nature. Bull of the WHO., 52: 393.

Reilly E.B., D.M. Kranz, S. Tonegawa, H.N. Eisen. 1986. A Functional gamma Gene Formed from Known gamma-gene Segments is not Necessary fro Antigen-Specific Responses of Murine Cytotoxic T Lymphocytes. Nature, 321: 878.

Reske-Kunz A.B., D. von Steldern, E. Rude, T. Diamanstein. 1986. Regulation of IL-2 Receptor Expression by Interleukin 2. Scand. J. Immunol., 23: 693.

Ritz J., T.J. Campen, R.E. Schmidt, H.D. Royer, T. Hercend, R.E. Hussey, E.L. Reinherz. 1985. Analysis of T-Cell Receptor Gene Rearrangement and Expression in Human Natural Killer Clones. Science. 228: 1540.

Riviere Y., P.J. Southern, R. Ahmed, M.B.A. Oldstone. 1986. Biology of Cloned Cytotoxic T Lymphocytes Specific for Lymphocytic Choriomeningitis Virus. V: Recognition is Restricted to Gene Products Encoded by the Viral S RNA Segment. J. Immunol., 136: 304.

Rosenthal K.L., J.R. Smiley, S. South, D.C. Johnson. 1987. Cells Expressing Herpes Simplex Virus Glycoprotein gC but not gB, gD or gE are Recognized by Murine Virus-Specific Cytotoxic T Lymphocytes. J. Virol., 61: 2438.

Rosenthal K.L., M.B.A. Oldstone, H. Hengartner, R.M. Zinkernagel. 1983. Specificity of *in vitro* Cytotoxic T Cell Clones Directed Against Vesicular Stomatitis Virus. J. Immunol., 131: 475.

Roska A.K., P.E. Lipsky. 1985. Dissection of the Functions of Antigen-Presenting Cells in the Induction of T-Cell Activation. J. Immunol., 135: 2953.

Rowe W.P., F.A. Murphy, G.H. Bergold, J. Casals, J. Hotchin, K.M. Johnson, F. Lehmann-Grube, C.A. Minis, E. Traub, P.A. Webb. 1970. Arenoviruses: Proposed Name for a Newly Defined Virus Group. J. Virol., 5: 651.

Rupp F., G. Frech, H. Hengartner, R.M. Zinkernagel, R. Joho. 1986. No Functional Gamma-Chain Transcripts Detected in an Alloreactive Cytotoxic T-Cell Clone. Nature, 321: 876.

Samelson L.E., T. Lindsten, B.J. Fowlkes, P. van den Elsen, C. Terhorst, M.M. Davis, R.N. Germain, R.H. Schwarts. 1985. Expression of the T-Cell Antigen Receptor Complex in Precursor Thymocytes. Nature, 315: 765.

Schmid D.S., J.P. Tite, N.H. Ruddle. 1986. DNA Fragmentation: Manifestation of Target Cell Destruction Mediated by Cytotoxic T-Cell Lines, Lymphotoxin-Secreting Helper T-Cell Clones, and Cell Free Supernatants. PNAS (USA), 83: 1881.

Schwartz R.H. 1987. Antigen Presentation. Fugue in T-Lymphocyte Recognition. Nature, 326: 738.

Simrell C.R., P.A. Klein. 1979. Antibody Responses of Tumor-Bearing Mice to their own Tumors Captured and Perpetuated as Hybridomas. J. Immunol., 123: 2386.

Steinmetz M., Z. Dembic. 1986. Organization, Rearrangement, and Diversification of Mouse T-Cell Receptor Genes. Curr. Top. in Microbiol. and Immunol., 126: 46.

Sung K-L.P., L.A. Sung, M. Crimmins, S.J. Burakoff, S. Chien. 1986. Determination of Junction Avidity of Cytolytic T-Cell and Target Cell. Science, 234: 1405.

Townsend A.R.M., J. Bastin, K. Gould, G.G. Brownlee. 1986. Cytotoxic T Lymphocytes Recognize Influenza Haemagglutinin that Lacks a Signal Sequence. Nature, 324: 575.

Townsend A.R.M., J. Rothbard, F.M. Gotch, G. Bahadur, D. Wraith, A.J. McMichael. 1986. The Epitopes of Influenza Nucleoprotein Recognized by Cytotoxic T Lymphocytes Can be Defined with Short Synthetic Peptides. Cell, 44: 959.

Townsend A.R.M., F.M. Gotch, J. Davey. 1985. Cytotoxic T Cells Recognize Fragments of the Influenza Nucleoprotein. Cell, 42: 457.

Townsend A.R.M., A.J. McMichael, N.P. Carter, J.A. Huddleston, G.G. Brownlee. 1984. Cytotoxic T Cell Recognition of the Influenza Nucleoprotein and Haemagglutinin Expressed in Transfected Mouse L Cells. Cell, 39: 13.

Townsend A. 1985. Molecules at Work on the T-Cell Surface. Immunol. Today, 6: 68.

Trapido H., C. Sanmartin. 1971. Pichinde Virus: A New Virus of the Tacaribe Group from Columbia. Am. J. Tropical Med. and Hyg., 20: 631.

Trinchieri G., B. Perussia. 1984. Human Natural Killer Cells: Biologic and Pathologic Aspects. Lab Invest., 50: 489.

Utsunomiya N., M. Nakanishi. 1986. A Serine Protease Triggers the Initial Step of Transmembrane Signalling in Cytotoxic T Cells. J. Biol. Chem., 261: 16514.

Utsunomiya, M. Tsuboi, M. Nakanishi. 1986. Interleukin 2 Increases T Lymphocyte Membrane Mobility Before the Rise in Cytosolic Calcium Concentration. Biochem., 25: 2582.

Vezza A.C., P. Cash, P. Jahrling, G. Eddy, D.A.L. Bishop. 1980. Arenavirus Recombination: the Formation of Recombinants Between Prototype Pichinde and Pichinde Munchique Viruses and Evidence that Arenavirus S RNA Codes for N Polypeptide. Virol., 106: 250.

Vezza A.C., G.P. Gard, R.W. Compans, D.A.L. Bishop. 1977. Structural Components of the Arenavirus Pichinde. J. Virol., 23: 776.

Vohr H.W., T. Hunig. 1985. Induction of Proliferative and Cytotoxic Responses in Resting Lyt-2+ T-Cells with Lectin and Recombinant Interleukin 2. Eur. J. Immunol., 15: 332.

Wagner H., C. Hardt, B.T. Rouse, M. Rollinghoff, P. Scheurich, K. Pfizenmaier. 1982. Dissection of the Proliferative and differentiative Signals Controlling Murine Cytotoxic T Lymphocyte Responses. J. Exp. Med., 155: 1876.

Walker C.M., V. Paetkau, W.E. Rawls, K.L. Rosenthal. 1985. Abrogation of anti-Pichinde Virus Cytotoxic T Cell Memory by Cyclophosphamide and Restoration by Coinfection or Interleukin 2. J. Imm., 135: 1401.

Walker C.M., W.E. Rawls, K.L. Rosenthal. 1984. Generation of Memory Cell-Mediated Immune Responses After Secondary Infection of Mice with Pichinde Virus. J. Immunol., 132: 469.

Weinberger O., S.H. Herman, J.L. Greenstein, M.F. Mescher, S.J. Burakoff. 1985. The Ability of Ia and H-2K^k-Bearing Membranes to Replace the Antigen-Presenting Cell in an H-2K^k Allogeneic Cytotoxic T Cell Response. Eur. J. Immunol., 15: 1013.

Yannelli J.R., J.A. Sullivan, G.L. Mandell, V.H. Engelhard. 1986. Reorientation and Fusion of CTL Granules after Interaction with Target Cells as Determined by High Resolution Cinemicrography. J. Immunol., 136: 377.

Young J.D.E., Z.A. Cohn. 1986. Cell-Mediated Killing: A Common Mechanism? Cell, 46: 641.

Young J.D.E., L.G. Long, C-C. Lieu, A. Dameano, D.A. Wall, Z.A. Cohn. 1986. Isolation and Characterization of a Serine Esterase from Cytolytic T-Cell Granules. Cell, 47: 183.

Young J.D.E., E.R. Podack, Z.A. Cohn. 1986a. Properties of Purifies Pore-Forming Protein (Perforin 1) Isolated from H-2 Restricted Cytotoxic T Cell Granules. J. Exp. Med., 164: 144.

Young J.D.E., Z.A. Cohn, E.R. Podack. 1986b. The Ninth Component of Complement and the Pore Forming Protin (Perforin 1) from Cytotoxic T Cells: Structural and Immunological and Functional Similarities. Science, 233: 184.

Zarling J.M., P.A. Moran, L.A. Lasky, B. Moss. 1986. Herpes Simplex Virus - Specific Human T Cell Clones Recognize HSV Glycoprotein gD Expressed by a Recombinant Vaccinia Virus. J. Virol., 59: 506.

Zeller W., M. Bruns, F. Lehmann-Grube. 1986. Viral Nucleoprotein can be Demonstrated on the Surface of Lymphocytic Choriomeningitis Virus-Infected Cells. Med. Microbiol. Immunol., 175: 89.

Zinkernagel R.M., C.J. Pfau, H. Hengartner, A. Althage. 1985a. Susceptibility to murine Lymphocytic Choriomeningitis maps to Class I MHC Genes - A Model for MHC/Disease Associations. Nature, 316: 814.

Zinkernagel R.M., T. Leist, H. Hengartner, A. Althage. 1985b. Susceptibility to Lymphocytic Choriomeningitis Virus Isolates Correlates Directly with Early and High Cytotoxic T Cell Activity, as well as with Footpad Swelling Reaction, and all three are Regulated by H-2D. J. Exp. Med., 162: 2125.

Zinkernagel R.M., K.L. Rosenthal. 1981. Experiments and Speculation on Anti-Viral Specificity of T-Cells and B-Cells. Immunol. Rev., 58: 131.

Zinkernagel R.M., G. Kreeb, A. Althage. 1980. Lymphohemopoietic Origin of the Immunogenic, Virus-Antigen-Presenting Cells Triggering Anti-Viral T-Cell Responses. Clin. Immunol. and Immunopathol., 15: 565.

Zinkernagel R.M. 1978. Major Transplantation Antigens in Host Responses to Infection. Hosp. Practice, July: 83.