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MAPPING THE CYTOTOXIC T-LYMPHOCYTE EPITOPES OF PICHINDE VIRUS

A Thesis Submitted to the
School of Graduate Studies
University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of
Master of Science
Department of Microbiology and Immunology
Faculty of Medicine

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ISBN 0-315-95906-1

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ABSTRACT

One of the aims of this study was to determine whether the glycoproteins of Pichinde virus harbour any cytotoxic T-lymphocyte (CTL) epitopes on the murine haplotypes, H-2^b and H-2^d, since, on neither of these MHC backgrounds are there any CTL epitopes on the nucleoprotein of this virus. Using a vaccinia virus recombinant, vvGPC, which expresses the full-length glycoprotein precursor (GPC) of Pichinde virus, standard chromium release CTL assays were performed. Three independent assays are shown for each of the haplotypes. In each of these assays for both of the haplotypes, it was observed that CTL derived against Pichinde virus did not recognize vvGPC-infected target cells nor did CTL derived against vvGPC recognize Pichinde-infected target cells. This indicates that no CTL were generated with either virus that might recognize the glycoproteins of Pichinde virus and, therefore, that the glycoproteins do not contain CTL epitopes on these murine MHC backgrounds. These results suggest that the epitopes responsible for the induction of anti-Pichinde CTL and for the recognition of Pichinde-infected cells on these haplotypes reside on the polymerase protein and/or the putative Z protein of this virus.

A second aim of this work was to compare the CTL epitopes of Pichinde wild-type virus with two temperature sensitive mutants derived from it. Both these mutants have been shown to be defective in their glycoprotein processing. Again, three assays are shown for each haplotype. The results found here indicate that on H-2^b, the wild-type virus and TS13 share the same epitopes as Pichinde-specific CTL recognize TS13

targets as well as Pichinde-infected targets. The H-2^d-restricted epitopes appear to be disrupted in TS13 as it is not recognized by Pichinde-specific CTL derived on this background. TS908 is not recognized by Pichinde-specific CTL on either haplotype suggesting that the wild-type virus' epitopes were probably disrupted during the derivation of this mutant.

ACKNOWLEDGEMENTS

I am grateful to my supervisor, Dr. Kathryn Wright, for her support, advice and encouragement during my stay in her laboratory. I would also like to thank the members of my thesis advisory committee: Drs. E. Brown, L. Filion, and I. Bernatchez-Lemaire for their guidance, and Dr. G. Wells who advised me on statistics and reviewed my statistical derivation.

Working in the department was an enjoyable experience for me thanks to the many people that I encountered and that I worked with. In particular, thanks to Essam, Denise, and Halina who made it fun to work in the lab. Thanks also to my fellow graduate students and to technicians, post-docs, profs, secretaries, etc. who made the department a friendly place.

I would also like to acknowledge the support and encouragement of my family and my friends. In particular, thanks to Kishna for his support.

I am grateful to MRC and to the University of Ottawa for funding me in this work.

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LIST OF ABBREVIATIONS

Å	Angstroms
BHK	baby hamster kidney cells
BLT	benzyloxycarbonyl-L-lysine thiobenzyl ester
CD4, CD8	T-cell surface markers
cpm	counts per minute
^{51}Cr	radioactive chromium-51
CTL	cytotoxic T-lymphocyte
dal	dalton
DMEM	Dulbecco's minimum essential media
E:T	effector to target ratio
FBS	fetal bovine serum
GPC	glycoprotein precursor
GP-1, GP-2	glycoproteins
H-2	nomenclature for the murine MHC locus
HEPES	N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid
IFN	interferon
IL	interleukin
kDa	kilodalton
L (protein)	large protein
L (segment)	large genome fragment
LCMV	lymphocytic choriomeningitis virus
MCMV	murine cytomegalovirus
MEM	minimum essential media
MHC	major histocompatibility complex
mL	millilitre
mM	millimolar
MOI	multiplicity of infection

nm	nanometre
NP	nucleocapsid protein
PBS	phosphate buffered saline
pfu	plaque forming unit
Pic	Pichinde virus
rpm	rotations per minute
RSV	respiratory syncytial virus
S	small genome fragment
^{35}S	radioactive sulfur-35
TCR	T-cell receptor
TS	temperature sensitive
μCi	microcurie
μg	microgram
μL	microlitre
v/v	volume per volume
vvGPC	recombinant vaccinia virus expressing the full length glycoprotein precursor of Pichinde virus
vSC8	control recombinant vaccinia virus
VSV	vesicular stomatitis virus
w/v	weight per volume
Z	zinc binding protein

INTRODUCTION

1. PICHINDE VIRUS

1.1 Arenaviruses

Pichinde virus was first isolated in the late 1960s from the rodent *Oryzomys albigularis* in the Pichinde valley in Colombia, South America. Using immune ascitic fluid derived against an early isolate of Pichinde, initial characterizations involving complement fixation and plaque-reduction neutralization tests and comparisons to other viruses revealed it to be a member of the New World or Tacaribe complex of the family *Arenaviridae*. Other observations made at the time, namely the virus' ability to asymptomatically persist in its host, the results of several animal studies, and later studies on the growth and stability of the virus further supported Pichinde's classification as an arenavirus (Trapido and Sanmartin, 1971; Mifune et al., 1971). Since this time, much has been learned about Pichinde and other arenaviruses and through these studies important advances in the areas of viral persistence and viral immunology have been made.

Presently, the family *Arenaviridae* comprises 16 members making it one of the smallest known families of RNA viruses. Arenaviruses have been divided into 2 groups. Initially this division was based on geographical distribution of the viruses and their serological and structural properties although the division has been subsequently confirmed by more detailed antigenic and genetic analyses. The New World or Tacaribe complex of arenaviruses includes Pichinde, Tacaribe, Amapari, Parana, Junin, Machupo, Flexal and Latino viruses isolated from mammals from various parts of South America and Tamiami virus isolated in Florida. Recently, two new viruses were added to this

group. Guanarito virus and callitrichid hepatitis virus were found in rodents from Venezuela and from the United States respectively (Salas et al., 1991; Stephenson et al., 1991). The Old World viruses were primarily isolated in Africa. Lassa, Mopeia, Ippy and Mobala make up this group. Lymphocytic choriomeningitis virus (LCMV), initially discovered in 1935, is also an Old World virus although it has a world-wide distribution. LCMV is considered the prototype of the arenavirus family (McCormick, 1990).

Arenaviruses are maintained in nature by a persistent infection of their vertebrate host. The natural hosts of most of these viruses are rodents, the exception being Tacaribe virus which is found in a species of bat. These persistent infections are characterized by an asymptomatic infection with viremia and viruria (Rawls et al., 1981). Neonatal transmission of the virus is believed to be the major mode of intraspecies transmission (McCormick, 1990). In the Pichinde valley, Pichinde virus was isolated from as many as 30% of the *O. albigularis* that were trapped. All of those studied showed viremia through the course of their lives (Trapido and Sanmartin, 1971).

Five arenaviruses have been found to be associated with human disease. In rural communities in South America, Machupo and Junin viruses are the etiological agents of Argentine and Bolivian hemorrhagic fevers respectively. A newly discovered virus, Guanarito virus, is responsible for a similar illness in Venezuela (Salas et al., 1991). In West Africa, Lassa virus causes Lassa fever. These four illnesses in humans manifest as severe, febrile illnesses with hemorrhagic consequences. They are often fatal especially if left untreated. Transmission of these viruses to humans is through contact

with infected rodents or their contaminated excretions (Peters, 1991). Human-to-human transmission has been documented in hospitals where Lassa fever patients have been treated (Frame et al., 1970; Peters, 1991). LCMV has also been shown to cause human disease. The illness ranges from asymptomatic or a mild flu-like illness in most cases to a severe illness with central nervous system involvement. The latter is rarely fatal although it usually results in a long convalescence. Like the other pathogenic arenaviruses, LCMV is transmitted to humans through contact with infected rodents although cases of laboratory acquired infections transmitted through contact with persistently infected cell cultures and animals have been documented (Oldstone, 1987; Peters, 1991). Pichinde virus is not known to cause human disease although anti-Pichinde antibodies have been detected in laboratory personnel working with high concentrations of the virus (Buchmeier et al., 1974). Pichinde infection of hamsters and guinea pigs has provided a safe animal model for the study of the human hemorrhagic fevers (Buchmeier and Rawls, 1977; Jahrling et al., 1981).

1.2 Virion structure

Pichinde virions are roughly spherical, pleomorphic particles that have a size of 50 to 300 nm (Mifune et al., 1971). This is typical of other arenaviruses which are quoted to be in this size range with an average diameter of 110 to 130 nm. The outer surface of the virion consists of an envelope derived from the host cell from which the virion buds (Murphy et al., 1975).

The nucleoprotein (NP) of Pichinde virus is the most abundant protein in the virion. Vezza et al. (1977) estimated the number of nucleoproteins to be 1500 per virion and roughly 60-70% of the total viral protein. NP is a 66 kDa non-glycosylated protein that is found in association with the 2 viral RNA species (Vezza et al., 1977). Young and Howard (1983) further defined this association by electron microscopy of the ribonucleoprotein complex released from purified virions. Their results show the NP as globular subunits 3 to 5 nm in diameter arranged 5 to 7 nm apart on long strands which fold into helical structures 12 nm in diameter.

Consistent with many other negative strand viruses, an RNA-dependent RNA polymerase activity has been found in purified Pichinde virus. This activity was shown to co-sediment with the viral ribonucleoprotein on a sucrose gradient (Carter et al., 1974; Leung et al., 1979). In 1981, Harnish et al. first reported the immunoprecipitation of a virus-coded 200 kDa, probably non-glycosylated, protein from virions and from infected cells. This L protein was presumed to be the RNA polymerase. Further support for this has come from sequencing data of the large genome fragment of two other arenaviruses, namely LCMV and Tacaribe. Both revealed an open reading frame coding for a similarly large (200-250 kDa) protein that had some sequence homology with known viral RNA polymerases (Singh et al., 1987; Salvato and Shimomaye, 1989; Iapalucci et al., 1989a).

The envelope of arenavirus particles contains viral glycoproteins which under electron microscopy appear as club-shaped projections extending 5 to 10 nm (Vezza et al., 1977). Although some arenaviruses appear only to have one glycoprotein,

Pichinde virus, like LCMV, contains 2 glycosylated proteins of sizes 52 and 36 kDa (Harnish et al., 1981). These are found in equimolar amounts in the envelope with an estimated 400 copies of each per virion (Vezza et al., 1977). Pulse-chase experiments have shown that these glycoproteins are produced from the post-translational cleavage of a 79 kDa precursor molecule (Harnish et al., 1981). GP-1 lies at the amino terminus of GPC whereas GP-2 occupies the remaining 231 amino acids at the carboxy end. The cleavage site is believed to be at 2 positively charged amino acids, arginine and lysine, at amino acids 271 and 272 (Buchmeier et al., 1987).

Little is known of the structure of the glycoproteins in the Pichinde virus envelope although recent studies in LCMV indicate that the larger GP-1 is a peripheral protein and that GP-2 is an integral membrane protein (Burns and Buchmeier, 1991). The report also concludes that the glycoproteins are assembled as homotetramers and that no detectable linkage occurs between GP-1 and GP-2.

Cloning and sequencing of the large genome segments of LCMV and Tacaribe has revealed an open reading frame which codes for a sixth viral protein in these viruses (Iapalucci et al., 1989b; Salvato and Shimomaye, 1989). Called p11 or Z as a result of its size, 10-11 kDa, and the presence of a zinc binding motif, this protein has been associated with the ribonucleoprotein complex in LCMV and has been shown to play an as yet unspecified role in both mRNA synthesis and RNA replication in Tacaribe (Salvato et al., 1992; Garcin et al., 1993). A protein of size 10-15 kDa has been observed in Pichinde infected cells although this may be a degradation product of NP (Ramos et al., 1972; Vezza et al., 1977; Harnish et al., 1981; Young and

Howard, 1983). Further evidence of a Z protein in this virus awaits the cloning of Pichinde's L segment.

Electron microscopy of thin sections of Pichinde virions has revealed 20 to 30 nm electron dense granules in the virions that are indistinguishable in size and shape to cellular ribosomes (Vezza et al., 1977). RNA analysis of the virions supported the idea that these are ribosomes and that they are derived from the host cell (Carter et al., 1973; Farber and Rawls, 1975; Vezza et al., 1978). Other arenaviruses have shown a similar sandy appearance due to ribosomes under electron microscopy. In fact, it was this feature that gave rise to the original family name, arenovirus, derived from the latin 'arenosus' meaning sand-like. The importance of these ribosomes in the replicative cycle of Pichinde is not known. The ribosomes are apparently functional (Chinault et al., 1981) but studies with virions containing TS ribosomes indicate that they are not required for the replication of the virus (Leung and Rawls, 1977).

1.3 Virus genome

Pichinde, like other arenaviruses, has a genome that consists of 2 single-stranded RNA species (Vezza et al., 1978). These segments, designated S and L for small and large, have estimated molecular weights of 1.3×10^6 and 2.7×10^6 dal respectively in Pichinde (Ramsingh et al., 1980). The formation of reassortants between wild-type Pichinde virus and wild-type Munchique virus, an isolate of Pichinde, was used to determine that the S segment codes for the nucleoprotein and the glycoprotein precursor whereas the L segment codes for the 200 kDa L protein (Vezza et al., 1980;

Harnish et al., 1983). Sequencing of the L segments of LCMV and Tacaribe indicated that the large segment also codes for the Z protein (Iapalucci et al., 1989b; Salvato and Shimomaye, 1989). It has subsequently been shown that NP and L are coded at the 3' end of the viral RNA while GPC and Z are at the 5' terminus (Auperin et al., 1984; Salvato and Shimomaye, 1989).

Sequencing of the entire S segment of Pichinde virus in 1984 revealed a unique characteristic in the coding strategy of this virus. The sequence indicated that the nucleoprotein is coded in a mRNA complementary to the 3' end of the viral RNA segment whereas GPC is coded in a mRNA complementary to the 3' end of the viral complementary RNA. This implies that although the mRNA for the NP can be made from the viral RNA, the mRNA for GPC can only be transcribed once the entire viral RNA has been replicated into the complementary strand (Auperin et al., 1984). This coding strategy is commonly referred to as ambisense coding and has been identified in all arenavirus segments thus far investigated including the L segments of LCMV and Tacaribe.

It has been hypothesized that the ambisense coding property of arenaviruses may play a role in the ability of these viruses to persistently infect cells and animals. In both cells and mice infected with LCMV a significant reduction in the glycoprotein was evident between acute and persistent infections although little or no change was observed for the nucleoprotein (Rawls et al., 1981; Oldstone and Buchmeier, 1982). Ambisense coding allows for the independent regulation of mRNA production for these two genes on the S segment and therefore may be involved in this effect.

Between the termination codons of the two genes coded on the S segment of Pichinde there is an intergenic region of 87 nucleotides. One prominent characteristic of this region is the potential for a hairpin structure stabilized by the base pairing of 14 guanines and cytosines and 4 adenines and uracils (Auperin et al., 1984). Similar guanine-cytosine rich hairpin structures, although varying in number, have been predicted in the intergenic regions of other arenaviruses and it is proposed that this secondary structure may serve as transcription termination signals for both genes (Franze-Fernandez et al., 1987; Ghiringhelli et al., 1991; Wilson and Clegg, 1991). If this is the transcription termination signal, it is not known how the polymerase can get by in order to replicate the viral RNA. Studies on the replication of Tacaribe virus showed that protein synthesis is required for the synthesis of viral and viral complementary RNA. This has led to the suggestion that a virus-coded protein, possibly the nucleoprotein, is required to bind to the intergenic region to allow replication to occur (Franze-Fernandez et al., 1987).

Another feature of the Pichinde virus genome that is common to other arenaviruses studied is the complementarity between the ends of the genome segments. The 19 nucleotides at the 3' and 5' ends of Pichinde S RNA are complementary with 2 mismatches (Auperin et al., 1984). This nucleotide sequence including the mismatches are conserved among several arenaviruses studied and may represent a common transcription start signal. The complementarity of the genomic ends could result in stable pan-handle forms of the viral RNA. Circularized RNA strands have been viewed

under electron microscopy for several arenaviruses including Pichinde (Vezza et al., 1978; Young and Howard, 1983).

2. CYTOTOXIC T-LYMPHOCYTES

Cytotoxic T-lymphocytes (CTL) are an important component of the cell-mediated arm of the immune system. As the name implies, cytotoxic T-cells lyse target cells. Altered cells such as cells infected with a virus or an intracellular parasite, and tumour cells have all been shown to be possible targets for CTL (Braciale, 1977; Anichini et al., 1987; Farrell et al., 1989). Most CTL display the CD8 T-cell surface marker and, by way of the T-cell receptor (TCR), recognize peptides from endogenously synthesized proteins that are presented in conjunction with the major histocompatibility (MHC) class I glycoproteins found on the surface of most body cells. Less common are $CD4^+$ cytotoxic T-cells. These cells recognize peptides presented by the MHC class II molecules and may be important in clearing infections by some viruses such as measles (Jacobson et al., 1989). This recognition of the MHC molecules by the TCR results in the phenomenon of MHC-restriction which is characteristic of CTL. CTL will only lyse cells presenting antigen in association with self-MHC molecules (Zinkernagel and Welsh, 1976). Similarly, due to the specificity of the TCR for the peptide, the lysis of the target is also antigen-specific. For example, CTL generated against a virus will only lyse cells infected with that virus or a cross-reactive one (Townsend and Skehel, 1984; McIntyre et al., 1985). This review will discuss the generation, recognition and effector functions of these immune cells. It will focus

mainly on virus-specific $CD8^+$ T-cells although many of the points will apply to the other types as well.

The major role of CTL in the immune system is one of immunosurveillance. In virus infections, CTL act by destroying cells which are infected with virus, a process which can occur before antigenic proteins appear on the cell membrane to trigger a humoral response. The importance of CTL in virus clearance has been documented for many different viruses, mostly in the murine system. These reports deal primarily with the adoptive transfer of primed or memory $CD8^+$ CTL into infected, often immunodeficient, mice resulting in virus clearance and recovery from infection. Reddehasse et al. (1988) examined the role of CTL in murine cytomegalovirus (MCMV) infection in immunocompromised mice by this method. These researchers showed that primed or memory $CD8^+$ CTL had a protective effect when administered either before or after virus inoculation. This was demonstrated by a decrease in virus titre as compared to untreated controls. Other experiments showed that $CD4^+$ T-cells were not involved in this effect. Jonjic et al. (1989) substantiated the role of CTL in MCMV infection by showing that $CD8^+$ CTL could control MCMV persistence in mice which had been depleted of $CD4^+$ cells. Adoptive transfer experiments with CTL in murine Herpes simplex virus type I (HSV-I) infection showed that these cells protected mice from lethal infection and mediated recovery from infection (Sethi et al., 1983; Larsen et al., 1983). Similarly, experiments with influenza, rotavirus and respiratory syncytial virus revealed the importance of these immune cells in resolving virus infection and virus-associated illness in mice (Yap and Ada, 1978;

Dharakul et al., 1990; Offit and Dudzik, 1990; Cannon et al., 1987). In most of these cases, virus clearance was found to be associated mostly with $CD8^+$ CTL and generally independent of the $CD4^+$ T-cell response.

Further evidence for the importance of CTL in murine viral infections has come from studies in mice with impaired CTL function. Hou et al. (1992), studying Sendai virus, showed that depletion of the $CD8^+$ cells *in vivo* using a monoclonal antibody against CD8 delayed virus clearance. A similar delay in the resolution of infection was observed when transgenic mice with a disruption in the β_2-microglobulin gene of the MHC class I complex were used. These mice were shown to have no mature $CD8^+$ T-cells. Reports for influenza virus and LCMV also indicated that mice lacking $CD8^+$ cells as a result of a disruption of this β_2-microglobulin gene, showed delayed virus clearance. As well, a higher mortality in influenza infected mice was observed and infection with LCMV appeared to result in virus persistence (Bender et al., 1992; Lehmann-Grube et al., 1993).

Although not as well studied, there is evidence that CTL are important in recovery from viral infections in humans. Quinnan et al. (1982) showed that transplant recipients who survived cytomegalovirus (CMV) infection all developed CMV-specific CTL responses while, in the cases of fatal CMV infection, little if any anti-CMV CTL were detected. CTL were also shown to play a role in human influenza infection. McMichael et al. (1983) inoculated human volunteers with a strain of influenza and found that effective virus clearance correlated with a demonstrable CTL response.

2.1 Generation of CTL

Through work done primarily in the murine system, a general model for the induction of CTL can be proposed. The first step involves the recognition and binding of the precursor CTL to the infected target cell. The MHC class I molecules of the latter cell contain peptides derived from the viral proteins and this MHC/peptide complex will be recognized by the TCR of the CTL. Other surface molecules of these two cells have also been implicated in this binding and may play a role in T-cell activation. These include the CD8 molecule which recognizes the MHC glycoproteins, CD2 and LFA-1 on the T-cell and LFA-3 and ICAM-1 on the target cell (Hughes et al., 1990). One result of activation at this time is the increase in cytokine receptors, most notably the receptor for IL-2, on the cell surface of the CTL. The second signal delivered to the T-cells is by way of these cytokines. Most of these will likely originate from T-helper cells which are activated by way of MHC class II antigen presentation by antigen presenting cells. These cytokines prompt the precursor CTL to proliferate and differentiate into fully functional CTL (Bertagnolli et al., 1991).

The requirement for helper T-cells is a disputed aspect of primary CTL induction. Several reports suggest that these cells are required for *in vitro* induction of CTL although in some cases they can be replaced in the cultures by lymphokines (Macatonia et al., 1991; Nonacs et al., 1992). Other reports show that $CD4^+$ T helper cells are not required *in vitro* especially if dendritic cells, presenting via class I molecules to CTL, are used as the stimulators (Boog et al., 1988; Young and Steinman, 1990). These reports suggest that the cytokines released by the cytotoxic T-cells may

be enough to stimulate their own proliferation and differentiation. There have also been several *in vivo* studies where $CD4^+$ cells were depleted in mice either by an anti-CD4 antibody or a germline mutation in the CD4 gene. In testing the CTL response in these mice, the researchers found that the depletion did not cause a disruption in the ability of these mice to generate a CTL response although the antibody response was dramatically decreased (Buller et al., 1987; Ahmed et al., 1988; Rahemtulla et al., 1991).

One requirement in the induction and activation of CTL that is generally agreed upon is that of lymphokines. These may be from any T-helper cells or macrophages involved in the induction or they may be from the CTL themselves. One well characterized cytokine is interleukin-2 (IL-2) which is released from T-cells and can function in an autocrine or paracrine manner as a signal for proliferation. Removal of IL-2 from *in vitro* culture or *in vivo* impaired CTL development (Gillis et al., 1981; Granelli-Piperno et al., 1984). IL-2, as well as some other interleukins, has also been shown to induce the production of perforin and serine esterases in murine CTL clones (Liu et al., 1990). These compounds are believed to play a role in cell-mediated lysis. Other lymphokines have also been postulated to be involved in the generation of the CTL response. These include IL-1 released from macrophages, IL-3, IL-6, and IFNγ (Curtsinger and Fan, 1984; Galandrini et al., 1991; Morris et al., 1982; Wille et al., 1989). As well as being involved in generating a CTL response, some of the cytokines, namely IFNγ, have been associated with direct anti-viral effects (Ramsay et al., 1993).

2.2 Recognition of infected cells by CTL

One key aspect of CTL function is the recognition of infected cells. This is necessary both in the delivery of the first signal in the induction of CTL and in the effector phase during which CTL lyse infected cells.

Formerly it was thought that CTL recognized full-length protein on the target cell surface. It was soon realized that this was not the case as cells expressing truncated versions of the proteins were effectively lysed by CTL. When peptides were used to sensitize target cells for lysis, it became apparent that the recognition elements were, in fact, very short amino acid sequences (Townsend et al., 1986). It is now known that the CTL epitopes of a protein are short peptides that are bound to the MHC class I molecules and presented on the cell surface. The T-cell receptor (TCR) and CD8 molecules of the CTL recognize the MHC/peptide structure in an antigen-specific, MHC-restricted manner (Kane et al., 1989; O'Rourke and Mescher, 1993; Romero et al., 1993).

The peptides presented in this manner with MHC class I molecules are derived from endogenously synthesized proteins, be they self or viral proteins. As the proteins are translated in the cytoplasm, protease digestion can occur (Goldberg and Rock, 1992) and the resulting peptides are transported in to the endoplasmic reticulum by specialized transporters (Barinaga, 1990). In the endoplasmic reticulum, the peptides become bound to the MHC molecules, the binding ability of each peptide being dependent on the MHC molecule (Falk et al., 1990; Rohren et al., 1993; Rojo et al., 1993). At this time further processing may take place (Rotzschke and Falk, 1991). The peptide/MHC

complexes are then transported to the cell membrane where they can be monitored by circulating T-cells.

Much insight has been gained into the recognition of target cells by CTL as a result of the elucidation of the structure of the MHC class I molecules. X-ray crystallography of several murine and human MHC class I molecules has revealed a long groove on the top of the molecules which can accommodate the bound peptide. This groove is formed by 2 α-chains running parallel about 10 Å apart over the top of a ß-pleated sheet which forms the base of the groove (Tsomides and Eisen, 1991). Much of the polymorphism among MHC molecules is a result of amino acid changes in the area of this groove (Bjorkman et al., 1987).

The data from the crystallography as well as the elucidation of several viral epitopes and the sequencing of peptides eluted from MHC molecules has revealed common characteristics among the MHC binding peptides. Typically these peptides were found to be 8 or 9 amino acids long. Usually 2 or 3 of these amino acids near the termini are responsible for the peptide binding to the MHC molecule as indicated by consensus allele-specific motifs derived from the sequence analysis of known peptides (Rothbard and Taylor, 1988). Amino acid changes other than one or two conservative substitutions at these locations abrogated the peptides' ability to bind to the MHC molecule. The remaining amino acids are primarily responsible for the recognition by the TCR. Single amino acid substitutions in any of these positions can disrupt the recognition of the target cell by a T-cell clone (Hahn et al., 1992). Gairin and

Oldstone (1993) have recently shown that the secondary structure of the bound peptide is also important for T-cell recognition.

2.3 Effector functions of CTL

As previously mentioned, CTL function by killing virus-infected cells. This phenomenon is readily observed *in vitro* using such assays as the chromium release assay. Evidence supporting the lysis as being the main function of CTL *in vivo* is limited. Opponents would argue that the release of cytokines such as IFN and IL-2 by activated CTL may play the major antiviral role in animal systems. There are two main lines of evidence to support the specific lytic activity of CTL on their targets *in vivo*. First are experiments done by McIntyre et al. (1985) who showed that adoptive transfer of Pichinde virus immune cells reduced Pichinde virus titres in mice infected with both Pichinde and LCMV, without affecting the LCMV titre. Similarly when LCMV immune cells were transferred, LCMV titres but not Pichinde titres were reduced. This indicates that the CTL activity is virus-specific *in vivo*, an effect that would not be observed if the cytokines were the main mediators. This evidence supported the earlier results of Lukacher et al. (1984) who performed similar experiments with influenza. The second line of evidence is exemplified by the work of Welsh et al. (1990) who showed that murine $CD8^+$ LCMV-specific CTL generated *in vivo* had 10 times the serine esterase activity than cells from normal mice. The serine esterase was believed to be contained within granules and was released from the CTL when they encountered their target. Competitive substrates for these proteases blocked target cell-lysis. Similar serine

esterase activity was found with other viruses as well. In a similar line of experiments, Young et al. (1989) showed that virus infection induced the production of perforin in CTL. Perforin, like serine esterases, is also believed to be involved in cell-mediated lysis.

There are two main models to explain the CTL induced lysis of target cells. The granule exocytosis model describes the release of proteins contained within cytoplasmic granules in the activated CTL. The proteins are released into the extracellular space between the CTL and its target cell. One of the proteins described in the granules is perforin or pore-forming protein. In the presence of Ca^{2+} this protein polymerizes resulting in the formation of ring-like structures 6 to 16 nm in diameter in lipid bilayer membranes (Masson and Tschopp, 1985). The target would then be lysed by osmotic rupture. Other contents of the granules include a series of serine esterases (Masson and Tschopp, 1987; Takayama et al., 1987). The exact substrates of these proteins is not known although inhibitors to these proteases affect the cell-mediated lysis.

The second model is that of lymphocyte triggered apoptosis of the target cell. One of the signs of this is DNA fragmentation in the target cell. Although some of the granule contents including some of the serine esterases have been shown to induce target cell DNA fragmentation (Hayes et al., 1989; Liu et al., 1989; Shi et al., 1992), it is believed that this mechanism can be induced by granule-independent means as well. A proposed route is the induction of Ca^{2+} in the target cells which results in the activation of enzymes such as endonucleases which may be responsible for apoptosis (Duke et al., 1983; Tirosh and Berke, 1985).

Although there is much support for these two models of cell-mediated lysis, there have been reports of CTL lysis that is Ca^{2+} independent and exocytosis independent, two conditions crucial to these models (Young et al, 1987; Ostergaard and Clark, 1989; Trenn et al., 1987). Other mechanisms to account for these and other results have been proposed although more evidence is needed to substantiate these proposals.

3. MAPPING THE CTL EPITOPES OF A VIRUS

Mapping the CTL epitopes of a virus means to identify the peptides within the viral proteins that bind to particular class I MHC molecules and render an infected cell susceptible to lysis by specific CTL as well as generate CTL specific for the peptide and for the virus. In the past, work done to elucidate viral epitopes has provided much information regarding the recognition element of CTL as well as the role of the MHC and antigen processing and presentation in the immune response. As previously mentioned, it was initially thought that CTL recognized full-length protein on the surface of infected cells. It was therefore surprising when internal viral proteins such as the nucleoprotein were found to be recognized by CTL. Also proteins with large deletions or truncations were effectively presented to CTL. The use of peptides in studying CTL resulted in the determination that the epitopes were actually short amino acid sequences potentially less than 10 amino acids long. These results and results from investigations into MHC class I structure and into antigen processing have prompted

a deeper understanding of how virus-specific CTL are generated and how CTL recognize virus-infected cells.

In the future, work done to define viral CTL epitopes will better this understanding and lead to further comprehension of the immune response against specific viruses. As consensus sequences of MHC binding peptides and the roles of the amino acids in and flanking the epitopes are further defined, it should become possible to effectively predict viral epitopes without extensive work. Since CTL play a role in clearing virus infections, infections by intracellular bacteria and parasites and in the immunosurveillance of tumours, improved understanding of their function will result in improved vaccines and treatment in these areas.

3.1 Common techniques

Most studies investigating viral CTL epitopes utilize several common techniques. The first of these techniques to be discussed is the chromium release assay. This assay, introduced in 1968 by Brunner et al., has become the standard method of observing cytolysis. The principle behind this assay is that cells that are incubated in sodium chromate will take up the chromium and retain it within the cell. If the cell is lysed the chromium will be released. By using radioactive chromium-51, measuring the gamma radiation in the supernatant following incubation of target cells with the effector cells will give an idea as to the amount of chromium released and therefore the amount of cell lysis. Chromium release values are usually expressed as a percentage of the total chromium release at 100% lysis with allowances made for the background or

spontaneous release. Other CTL assays have been developed in the hope of overcoming the main disadvantage of the chromium assay, that of radioactivity (Mullbacher et al., 1984; Suhrbier et al., 1991; Provinciali et al., 1992). None of these has yet managed to replace the chromium release assay. The BLT assay measures the serine esterase released by activated CTL using an esterase substrate, BLT, in a colorimetric reaction. This assay has been used to study the effector functions of CTL and may present the most viable alternative to the chromium release.

In order to study the immune response against a particular viral protein, it is necessary to separate that protein from the rest of the virus. For this reason a viral expression vector is very useful. Vaccinia virus, a member of the poxvirus family, has been shown to be able to accommodate up to 25,000 base pairs of exogenous DNA while retaining its infectivity. This plus the facts that the virus has a large host range and that it has a cytoplasmic replication cycle thus avoiding nuclear modifications such as RNA splicing, has made vaccinia virus a popular expression vector (Moss, 1992).

Recombinant vaccinia viruses have been widely used by viral immunologists who wish to study the immune response against the viral components. This has prompted the identification of many B and T cell epitopes. In CTL studies, recombinant vaccinia viruses have been used both to sensitize target cells for lysis by virus-specific CTL and also to immunize animals to generate CTL. Deleted and truncated proteins as well as the full length versions have been used in these studies. Just recently, Whitton et al. (1993) reported the construction and use of a recombinant vaccinia virus

expressing 2 CTL epitopes from 2 different proteins of LCMV linearly arranged within the space of 64 nucleotides.

For CTL studies, the effector cells can be derived directly by primary immunization of animals or by secondary stimulation *in vitro* of immune cells from previously infected mice or humans. As a result of being able to restimulate the cells *in vitro*, it has been possible to maintain CTL lines in the laboratory by periodic stimulation of existing CTL. Serial dilutions and stimulation of the resulting populations has allowed the development of CTL clones. These clones have been used to analyze the interaction of CTL of particular specificities with the specific epitope.

The use of synthetic peptides has also provided much information in the elucidation of CTL epitopes. Peptides are incubated with the target cells in the CTL assay such that the peptides bind externally to the MHC molecules of the cells. This external binding of the peptide to the MHC class I molecules bypasses the requirement of endogenous protein synthesis and intracellular antigen processing. With this method, peptides have been used to finely map the epitope once the general region containing the epitope is known. Peptides and mutated versions of the peptides have been used in MHC binding assays as well as with CTL clones in CTL assays in order to investigate the importance of each amino acid residue. This has yielded much information regarding MHC-binding motifs and acceptable substitutions and the fine specificities of the T-cell clones.

3.2 Viral CTL epitopes

Through the use of vaccinia virus recombinants expressing full-length and truncated versions of viral proteins as well the use of synthetic peptides, CTL epitopes for many viruses on several human and murine backgrounds have been defined. The results have lead to some generalizations regarding viral CTL epitopes. First of all, unlike humoral responses which are primarily directed against surface proteins of a virus, CTL epitopes are more commonly found on internal viral proteins such as the nucleoproteins. Although for some viruses on some backgrounds there is a CTL response directed against the outer glycoproteins, in general, there appears to be a response against the nucleoprotein as well. For example, LCMV-specific CTL are directed against the nucleoprotein on three murine haplotypes studied and only on one of these is there a response against the glycoprotein (Whitton et al., 1988a). Influenza virus CTL also exhibit a reactivity towards the nucleoprotein (Gould et al., 1989) although CTL epitopes have been found on the glycoproteins, HA and NA (Wysocka and Hackett, 1990; Sweetser et al., 1989). Kees and Krammer (1984) found that most influenza memory CTL react with epitopes on internal virus determinants rather than the glycoproteins. Other viruses such as vesicular stomatitis virus (VSV) and respiratory syncytial virus (RSV) have been found to have epitopes on the nucleoprotein with less or no reactivity against the glycoproteins (Bangham et al., 1986; Puddington et al., 1986).

Other internal proteins have also served as CTL targets. The matrix protein, the polymerases, PB1, PB2, and PA and the nonstructural protein, NS1, of influenza have

all been shown to have CTL epitopes on various MHC backgrounds (Bennink et al., 1987; Gotch et al., 1987; Cossins et al., 1993). For LCMV, there appears to a response directed against proteins coded for the L segment (L and Z proteins) (Hany et al., 1989; Lewicki et al., 1992). Nonstructural proteins of cytomegalovirus, Dengue virus, rabies virus, RSV, herpes virus as well as many others have been found to harbour CTL epitopes (Jonjic et al., 1988; Rothman et al., 1993; Larson et al., 1991; Cherrie et al., 1992; Banks et al., 1993).

One potential advantage of having CTL epitopes on internal proteins is that these proteins are generally more conserved among different virus strains. As a result, it comes as no surprise that CTL that are found to be cross-reactive with other virus strains are usually directed against the nucleoprotein. This is illustrated by studies on influenza which showed NP-specific CTL to be subtype specific whereas HA-specific CTL are most often strain specific (Yewdell et al., 1985).

Typically there are 0 to 2 epitopes per gene, each epitope restricted to one MHC molecule on a murine haplotype. For example, on H-2^d, 97% of LCMV-specific clones derived from immunized mice were found to be specific for one 9 amino acid epitope restricted to the L^d molecule (Whitton et al., 1989). In contrast, on H-2^b, a large proportion of the clones are specific for an epitope on GP-1 restricted to the D^b molecule (Whitton et al., 1988b). There is also another epitope on GP-1 restricted to D^b. Although most epitopes bind to only one MHC molecule, there have been reports of epitopes restricted to more than one molecule. Oldstone et al. (1992) reported an

LCMV epitope that is active on murine haplotypes, d, u, and q. This may reflect a similarity in the MHC structure.

4. CTL AND ARENAVIRUSES

Lymphocytic choriomeningitis virus (LCMV), the prototype of the arenavirus family, is the most extensively studied arenavirus and one of the most extensively studied viruses with respect to CTL. It was with this virus that it was first reported that CTL activity is virus-specific and MHC-restricted (Marker and Volkert, 1973; Zinkernagel and Doherty, 1974). CTL generated against LCMV in mice on a particular murine background were ineffective against MHC-mismatched LCMV-infected cells and against cells infected with another virus although they efficiently lysed syngeneic LCMV-infected cells. This virus-specificity and MHC-restriction of CTL has been subsequently confirmed for many other viruses and is now one of the major tenets in immunology. LCMV was also one of the first viruses investigated using CTL clones and synthetic peptides. These two techniques have resulted in much information concerning LCMV CTL epitopes as well as in the understanding of CTL activity and of CTL epitopes in general.

The role of CTL in LCMV infection in mice has been established through experiments such as those involving adoptive transfer. Zinkernagel and Welsh (1976) first showed that the transfer of immune spleen cells could mediate the clearance of virus *in vivo*. These results have since been substantiated in similar experiments by Bryne and Oldstone (1984) who transferred cloned CTL lines and

Moskophidis et al. (1987) who showed that clearance was mediated by $CD8^+$ T-cells. Virus-specific CTL transferred into mice have also been shown to clear persistent LCMV infections in these mice (Oldstone et al., 1986; Jamieson et al., 1987).

Also supporting a major role for CTL in LCMV infection are experiments in which vaccinia virus recombinants were used to protect mice against lethal LCMV inoculation. Hany et al. (1989), Schulz et al. (1989), and Klavinskis et al. (1989) all reported that vaccinia virus recombinants expressing the full-length or truncated nucleoprotein (NP) or glycoprotein (GPC) genes protected mice against subsequent lethal challenge of LCMV. This protection occurred only if the expressed protein contained a CTL epitope restricted to the murine background of the mice. More recently, it has been shown that expressing several LCMV CTL epitopes consecutively on a short protein in a recombinant vaccinia virus can also protect mice against virus challenge. These epitopes were from different genes and restricted to different haplotypes. This "string-of-beads" vaccine was shown to work on the different haplotypes and could prevent viral persistence as well as death in the mice (Whitton et al., 1993; Oldstone et al., 1993).

As with many other viruses, the nucleoprotein of LCMV appears to be the major target antigen in the anti-viral CTL response. Hany et al. (1989) showed that on 4 (b,d,q,s) out of 6 murine haplotypes, there was a CTL response directed against NP while on 2 (b,f) of these 6 haplotypes, there was a response directed against GPC. Some of these results confirmed results found by others (Joly et al., 1989; Whitton et al., 1988a,b; 1989). On the sixth haplotype, $H\text{-}2^k$, the response was not directed against the

nucleoprotein or the glycoproteins which suggests that it is directed against a product coded on the L segment (L or Z proteins). Lewicki et al. (1992) substantiated the results for H-2^k suggesting that the epitopes are on the polymerase. Contrary to Hany et al., these researchers found a response directed against the nucleoprotein on this haplotype as well.

The exact peptide that is the epitope has been determined for several of these epitopes, particularly for those on H-2^b and H-2^d. This was done using a series of vaccinia virus recombinants expressing truncated versions of the viral proteins and by using synthetic peptides to finely map the epitope once the region was known. Using cells transfected with a foreign MHC gene or recombinant cell lines expressing MHC alleles from different haplotypes allowed the determination of the restricting MHC molecule for each of these epitopes. The epitopes ranged in length from 5 to 9 amino acids, with the majority being 8 or 9. They are restricted to various MHC molecules and can be found in any region of the proteins (Klavinskis et al., 1990; Joly et al., 1989; Whitton et al., 1989).

There are several other areas of study which involve LCMV and CTL. First is the observation that the disease caused by the virus in mice, lymphocytic choriomeningitis, is a result of cell-mediated immunopathology. Adult mice inoculated intracerebrally with the virus show an increase in $CD8^+$ CTL in the cerebrospinal fluid and die within 7 days. Immunosuppression of these mice prevents death although it does allow the virus to persist (Allan et al., 1987). Also studied is the role of CTL in LCMV persistence. Adoptive transfer of virus-specific CTL has been shown to clear

persistent infection in mice (Oldstone et al., 1986; Jamieson et al., 1987). It has been observed that persistently infected mice have little or no CTL response to LCMV although anti-LCMV antibodies are detected. The role of CTL in persistence is now being investigated using a strain of LCMV isolated from the spleen of a persistently infected mouse. This virus variant which has an amino acid change in the glycoprotein, does not elicit CTL and can persist in an immunocompetent mouse (Salvato et al., 1991).

Much of the information regarding the CTL response against other arenaviruses comes from studies of animal models. It is believed that both cell-mediated and humoral responses play roles in the clearance of the viruses from the animals with cell-mediated immunity dominating with the Old World arenaviruses and the antibody response playing a greater role with the New World viruses, particularly with the hemorrhagic fever viruses in humans. Studies of several animal models have also shown that the disease in the animals and in humans is not a result of immunopathology as it is in the murine LCMV model (Peters et al., 1987).

With regards to Pichinde virus CTL response, there is little information. It has been determined that this virus elicits a strong cell-mediated response upon infection in mice. The primary response peaks around 7 days which is comparable to that found for LCMV (Walker et al., 1984; Whitton et al., 1989). A secondary CTL response has also been observed both after *in vivo* and *in vitro* stimulation. This response appears to peak after 4 days (Walker et al., 1984; Ozols et al., 1990). The CTL epitopes of Pichinde have yet to be determined. Ozols et al. (1990) found that a recombinant

vaccinia virus expressing the nucleoprotein of Pichinde could not elicit anti-Pichinde CTL nor could it sensitize targets for lysis by anti-Pichinde CTL. This was observed on three murine backgrounds. They concluded that there are no CTL epitopes on the Pichinde nucleoprotein on these haplotypes.

OBJECTIVES

In 1990, Ozols et al. reported that the nucleoprotein of Pichinde virus did not contain any CTL epitopes on the murine haplotypes, b, d, and k. These results came from experiments using 2 recombinant vaccinia viruses one expressing full-length NP, the other a truncated NP missing the first 51 amino acids. Based on CTL epitope analysis of another arenavirus, LCMV, as well as generalizations from other viruses, it was decided that the next logical proteins to examine for CTL epitopes on these MHC backgrounds were the glycoproteins.

Several temperature sensitive (TS) mutants of Pichinde virus have been derived which are known to have defects in the glycoprotein processing (Vezza and Bishop, 1977; Shivaprakash et al., 1988). It is possible that the amino acid changes in the glycoproteins or other proteins of these mutants may disrupt a CTL epitope of the wild-type virus. Therefore it is of interest to compare the CTL cross-reactivity of these viruses.

The objectives can be stated as follows:

1. To determine whether the glycoproteins (GP-1, GP-2) of Pichinde virus contain any CTL epitopes by using a recombinant vaccinia virus expressing full-length glycoprotein precursor in a CTL assay. This will be investigated two ways:

a. To observe if cells infected with the recombinant can serve as targets for Pic-specific CTL

b. To observe if the recombinant can prime mice for CTL specific for Pichinde

2. To investigate the cross-reactivity between Pichinde and two Pichinde TS mutants by observing the lysis of infected target cells by Pic-specific CTL.

MATERIALS AND METHODS

1. CELLS

Balb/c, MC57 and L929 cells are murine fibroblast cell lines of different MHC haplotypes. Balb/c cells (H-2^d) and L929 (H-2^k) were initially provided by Dr. K. Dimock (University of Ottawa, Ottawa, ON). MC57 cells are H-2^b and were provided by Dr. K. Rosenthal (McMaster University, Hamilton, ON). The African green monkey cell lines, Vero E6 and CV-1, were also used in these experiments. The Vero cells were initially provided by Dr. M. J. Buchmeier (The Scripps Research Institute, La Jolla, CA) while the CV-1 cells were provided by Dr. K. Dimock. A seed culture of baby hamster kidney cells (BHK) cells was initially supplied the Laboratory Centre for Disease Control (Ottawa, ON).

The murine cell lines and the Vero and CV-1 cells were passaged twice a week in 25 cm^2 plastic flasks (Corning Glass Works, Corning, NY) using, as media, Minimum essential media (MEM) supplemented with 7% (v/v) heat inactivated (56^{0}C for 30 min) fetal bovine serum (FBS), 0.23% (w/v) sodium bicarbonate, 100 units/mL penicillin, 100 μg/mL streptomycin (Penicillin-streptomycin solution), and 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY). The BHK cells were passaged twice in week in the Corning flasks with Dulbecco's MEM (DMEM; Gibco Laboratories). This media was supplemented with 10% FBS, 1% HEPES (1M), Penicillin-streptomycin as above, and 1% anti-PPLO (100x; Gibco Laboratories). For passaging of all cell cultures, phosphate-buffered saline (PBS; 0.01 M phosphate, 0.15 M NaCl, pH 7.2-7.4) was use to wash the monolayers. Trypsin-EDTA (10x; Gibco

Laboratories) was diluted in PBS and used to trypsinize the cells. The flasks were placed in a 37^0C, 5% CO_2 incubator to promote cell growth.

2. MICE

Two inbred strains of mice were used in this study. Balb/c mice are H-2^d and thus MHC-matched to the Balb/c cell line. C57Bl/6 mice are H-2^b and matched with the MC57 cell line. Both strains of mice were provided by Charles River Company (Montreal, PQ) and were maintained by the Animal Care Services, University of Ottawa. Newly infected mice were kept isolated for at least four days using cage filter tops or by being placed in separate areas of the housing unit.

3. VIRUSES

3.1 Pichinde virus: wild-type and TS mutants

The wild-type Pichinde virus used in this study is strain AN3739 initially provided by Dr. M. J. Buchmeier (The Scripps Research Institute, La Jolla, CA). The TS mutants, TS13 and TS908, were obtained from Dr. W. Rawls (McMaster University, Hamilton, ON). TS13 was initially derived and characterized by Vezza and Bishop (1977).

To grow the Pichinde wild-type and TS mutant stocks, confluent flasks of BHK cells in DMEM were infected at a MOI of 1. Wild-type infected cells were placed at 37^0C while the TS infected cells were placed at 34^0C for virus growth. The supernatants from the flasks were collected after 48 and 72 hours. The two supernatants for each

virus were combined and titrated. The stocks were aliquotted and stored at -80^{0}C. For virus titration, ten-fold dilutions of the stocks were used to infect the wells of 6-well plates (Costar, Cambridge, MA) of confluent Vero cells. 0.3 mL of inoculum was used per well with 2 wells infected for each dilution. After a one hour absorption period, an overlay of 1xMEM with 0.75% agar (Bacto/Difco) was added to the cells. The plates were then left for 4 days after which a second overlay was added. This overlay was the same as before except that 1.5% neutral red (Sigma, St. Louis, MO) was also included. On the fifth day, plaques could be seen. These were counted and averaged to determine the virus titre. Pichinde wild-type stocks were titrated at 37^{0}C while the TS stocks were titrated at both the permissive (34^{0}C) and non-permissive (39^{0}C) temperatures to ensure that the TS phenotype had been maintained.

3.2 Vaccinia virus recombinants: vvGPC and vSC8

vvGPC is the recombinant vaccinia virus expressing full-length Pichinde glycoprotein precursor. This virus was constructed in the laboratory by E. Wanas (Wanas, E., 1993). vSC8 is a control recombinant vaccinia virus which expresses the lacZ gene. It was provided to us by Dr. D. Harnish (McMaster University, Hamilton, ON) with the permission of Dr. B. Moss (National Institute of Allergy and Infectious Diseases, Bethesda, MD).

Stocks of both of these viruses were grown and titrated on CV-1 cells. Before infecting any cells with either virus, the virus was trypsinized. This was done by incubating the desired amount of virus in a solution of 1% trypsin-EDTA in media

without FBS in a 37^{0}C water bath for 15 minutes. To grow the virus stocks, trypsinized virus was used to infect confluent flasks of CV-1 cells. When the cytopathic effect was prominent (usually after 48-72 hours), the cells were scraped into the media which was then removed. The cells were pelleted and resuspended in approximately 2.5 mL MEM (supplemented as above except without FBS) per 75 cm^2 of cells. The virus stocks were then aliquotted and stored at -80^{0}C. To titrate the stocks, 0.3 mL of ten-fold dilutions of the stock were used to infect 6-well plates of CV-1 cells as per the titration of Pichinde virus. After one hour, an overlay made up of 1xMEM and 0.8% agarose (BDH Chemicals) was added. Two days later, a 10% formaldehyde-saline solution was added on top of the overlay and the plates left at room temperature overnight. The following day the formaldehyde and the overlays were carefully removed. The fixed cells were then stained with a 1% crystal violet dye solution after which the plaques could be counted and the titre determined.

4. IMMUNOPRECIPITATION OF GPC FROM INFECTED CELLS

Confluent 25 cm^2 flasks of MC57 cells were infected with wild-type Pichinde (MOI=1, 24 hrs) and with vvGPC and vSC8 (MOI=3, 6 hrs). One flask of uninfected cells was included as a negative control. To label the proteins, the cells were first starved by replacing the media with methionine-free DMEM media (ICN Biomedicals, Costa Mesa, CA) for the last hour of infection. Following this, 1 mL of methionine-free media supplemented with 100 μCi ^{35}S-methionine (Amersham, Oakville, ON) was placed in the flasks. One hour later this media was removed and the monolayers washed

3 times with PBS. Lysis buffer (1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 20 mM Tris, 137 mM NaCl, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$) was used to disrupt the cells. Approximately 1 mL of this buffer was placed in the flasks which were then placed on ice for 10 minutes. The cell lysates were then transferred to 1.5 mL eppendorf tubes and stored at -80^{0}C until required.

To set up the immunoprecipitation, Sepharose-coupled protein A (Sigma, CL-4B) was washed 3 times with lysis buffer with 10 mM aprotinin, 10 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM benzamidine hydrochloride and 1% (w/v) ovalbumin. A final wash was done with the same solution without ovalbumin. Cell lysates, pelleted to remove cell debris and equivalent in cell number to one flask of Pichinde infected cells, were mixed with 100 µL of the Sepharose beads and 10 µL of a monoclonal antibody specific for a common epitope of arenaviruses on GP-2. This antibody, 33.6, was kindly provided by Dr. M. J. Buchmeier. The tubes were rotated at 4^{0}C for 3 hours, following which the beads were pelleted and washed 5 times with wash buffer (100 mM Tris, 500 mM LiCl; pH 9.0). Sample buffer (0.125 M Tris, 2% SDS, 2% ß-mercaptoethanol, 20% glycerol, 0.05% bromophenol blue) was added to the washed beads and the beads were heated at 90^{0}C for 3 minutes. The samples were analyzed by SDS-polyacrylamide gel electrophoresis on a 10% gel (Laemmli, 1970). Electrophoresis was performed at 40 mA for 3 hours. To prepare the gel for autoradiography, it was fixed and then soaked in Amplify (Amersham, Oakville, ON) before being dried. The dried gel was then exposed to Kodak XRP film (Eastman Kodak Co., Rochester, NY) for 3 days at -80^{0}C.

5. CHROMIUM RELEASE ASSAY

5.1 Effector cells

The effector cells used in this study were secondary effectors. That is, memory CTL from mice were restimulated *in vitro* before being used in the assay. Preparation of the effectors was based on the methods used by Ozols et al. (1990) for Pichinde and Townsend and Skehel (1984) and Yewdell et al. (1985) for influenza. The media used for collecting the spleens and for resuspending and incubating the cells was filter sterilized RPMI 1640 supplemented with 10% (v/v) FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES (BDH Chemicals Ltd, Poole, UK), and 50 μM ß-mercaptoethanol (Bio-Rad Laboratories, Richmond, CA).

Mice were injected intravenously through a tail vein with $2x10^7$ pfu of virus, either Pichinde or vvGPC. No earlier than 3 weeks later, the infected mice and at least one syngeneic uninfected mouse were sacrificed and the spleens aseptically removed. In most cases, the spleens of two infected mice were combined to generate the Pic-specific or vvGPC-specific effectors. Cell suspensions were made of the spleens by grinding cut-up spleens with the piston of a 3cc syringe through a wire mesh placed over a 60 mm tissue culture dish (Corning Glass Works, Corning, NY). The cell solution was transferred to a 15 mL tube (Sarstedt, St. Laurent, PQ) and the mesh and dish washed with a further 5 mL of media. This was also transferred to the tube. The splenocytes were pelleted by centrifugation at 1000 rpm for 10 minutes (Sorvall^R GLC-2B centrifuge). The media was removed and a known volume of fresh media added. Viable cells were counted using trypan blue dye exclusion (EM Diagnostic

Systems, Gibbstown, NJ) and resuspended at a concentration of $2x10^7$ cells/mL. Red blood cells and dead cells were not included in the count. These cells are the responder cells.

Stimulator cells were splenocytes from an uninfected mouse. These cells were counted and divided into 2 or 3 tubes and precipitated by centrifugation before resuspension in media containing Pichinde virus (MOI=0.5) or trypsinized vvGPC (MOI=0.3) and incubation for 60 minutes at 37^0C. After the infection, media was added so that the final concentration of the stimulator cells was $4x10^6$ cells/mL.

To set up the secondary cultures, 0.5 mL of the responder cells (Pic or vvGPC) and 0.5 mL of the appropriate stimulator cells (Pic or vvGPC, respectively) were added to 9 mL of media in 25 cm^2 plastic flasks. The final concentration of the cells was then 10^7 responder cells and $2x10^6$ stimulator cells (5:1 ratio) in 10 mL of media. In some experiments, responder cells were incubated with uninfected stimulator cells as a control for the secondary stimulation. The flasks were incubated upright in a 37^0C, 5% CO_2 incubator for 5 days.

On the day of the assay, flasks with the same responder-stimulator pair were combined in several 50 mL tubes (Nunc, Denmark). These were spun to pellet the cells. The cells were then combined in one 50 mL tube by resuspending the cells from each tube in 2 mL media. The cell count in the resulting cell suspension was determined by trypan blue exclusion and the cells diluted to $5x10^6$ cells/mL. The effector cells were then ready to be used in the assay.

5.2 Target cells

The target cells used in the assays were syngeneic to the effectors except for the MHC control target. One confluent 25 cm^2 flask of cells was infected for each virus although, for the vaccinia targets, the wells of a 6-well plate were occasionally used instead. To infect the cells, the media was removed and the virus inoculum added: 1 mL/flask or 0.3 mL/well. The cells were then placed at 37^0C to allow virus absorption to occur. After one hour, fresh media was added and the flasks returned to the incubator. Wild-type Pichinde virus and the two TS mutants were infected at a MOI of 1 or greater for 24 or 48 hours. The TS mutant targets were kept at 34^0C for the duration of this period. The vaccinia targets were infected overnight at a MOI of 1 or, on the day of the assay, for 6 hours at a MOI of 3 or 5. A confluent flask of uninfected cells was used as the mock-infected control.

To label the targets for the CTL assay, the cells were trypsinized, washed once and pelleted. The media was poured off and the cells were resuspended in whatever media remained in the tube and 100-200 μCi of chromium-51 (NEN, Markham, ON; Amersham, Oakville, ON) was added. The tubes were placed at 37^0C for 90 minutes with occasional shaking to resuspend the cells.

At the end of the incubation, approximately 10 mL of media was added to each tube and the cells were washed 3 times in media. The cells were resuspended in 2 mL of media and the total cell count determined by staining with trypan blue. The cells were then diluted to a final concentration of 10^5 cells/mL.

5.3 CTL assay

In the assay, each effector was matched with various targets so as to include the appropriate controls. Pichinde-infected targets served as the positive control for Pic-specific effectors, while vSC8- and vvGPC-infected targets provided the positive control for the vvGPC-specific effectors. Mock cells were the negative control for both effectors. vSC8 infected targets were included as a control for vvGPC targets and as a virus control for Pic-specific effectors. In most experiments, an allogeneic cell line infected with Pichinde was included as an MHC control.

The assay was set up in 96-well V-bottom plates (DiaMed Lab Supplies Inc., Mississauga, ON). Each effector-target pair was done at 4 ratios resulting in final concentrations of $5x10^5$ (50:1), $2.5x10^5$ (25:1), $1.25x10^5$ (12.5:1), $62.5x10^4$ (6.25:1) effector cells with 10^4 target cells in 200 μL. Quadruplicate wells were set up for each ratio. Effectors were added to the wells first and serial dilutions in media performed to achieve the correct number of effectors in 100 μL in the wells. 100 μL (10^4 cells) of the labelled target cell solutions was then added to the wells.

Separately, 4 wells were set up with 100 μL of targets and 100 μL of media as a measure of the spontaneous chromium release. Another 4 wells were set up to measure maximum chromium release. These consisted of 100 μL of targets and 100 μL HCl.

The plates were incubated at 37^0C for 5 hours. At the end of this incubation, 100 μL was carefully pipetted off the top of each well and transferred to disposable 12x75 mm culture tubes (Baxter Healthcare Corporation, McGaw Park, IL). These tubes

were passed through a gamma counter (Beckman Gamma 5500 Counting System, Irving, CA) to measure the radioactivity in the samples. Counts from quadruplicate wells were averaged and the percent chromium released calculated according to the formula:

$$\%^{51}Cr\ Release = \frac{CPM_{sample} - CPM_{spon}}{CPM_{max} - CPM_{spon}} \times 100\%$$

where CPM_{sample} : average count for the wells of the effector-target pair at one ratio

CPM_{spon} : average count for the wells measuring spontaneous release for that target

CPM_{max} : average count for the wells measuring the maximum release for that target

5.4 Data handling and statistics

Chromium release values were calculated by hand or using the Microsoft Excel™ spreadsheet program. Graphs were plotted using Sigma Plot™. Appendix 1 shows the derivation of the formula used to calculate the 90% confidence intervals. These calculations were also done using Microsoft Excel™.

6. INFECTIOUS CENTRES ASSAY

Three mice were immunized intravenously with $2x10^7$ pfu with each of Pichinde wild-type virus and the Pichinde TS mutants. Two, three, or four days later the mice were sacrificed and their spleens removed. The spleens were prepared as per the effectors in the CTL assay. The cell number was determined using trypan blue exclusion and the cells adjusted to 10^6 cells/mL. Serial dilutions in media were done to create ten-fold dilutions of the cells. For each dilution, 2 wells of 6-well plates of Vero cells were infected with 0.3 mL. Two identical sets of plates were prepared for the TS mutants. The infection was allowed to occur for one hour following which an overlay consisting of a 1:1 mixture of 2xMEM supplemented with 10% FBS, 0.46% sodium bicarbonate and penicillin and streptomycin, and 1.5% agar was placed over the cells. The plates with wild-type virus were placed at 37^0C. For the TS mutants, 1 set of plates was placed at 34^0C, the other at 39^0C. Five days later a second overlay which included 2% neutral red was added to the wells. The following day plaques were counted and the number of infectious centres, expressed as plaques per 10^6 cells, was calculated.

RESULTS

1. VACCINIA EXPERIMENTS

Although the nucleoproteins of many viruses already studied have been shown to have CTL epitopes on various MHC backgrounds, Ozols et al. (1990) showed that Pichinde nucleoprotein harboured no such epitope. This was determined using 2 vaccinia virus recombinants, one expressing full-length NP, the other expressing NP minus the first 51 amino acids, in the standard chromium release assay. Three murine backgrounds, H-2^b, H-2^d, and H-2^k, were considered and on these backgrounds, Pic-specific effectors did not lyse cells infected with either of the recombinants nor could CTL specific for these viruses lyse Pic-infected targets. Pichinde virus is believed to have only 4 or 5 coded proteins and since CTL epitopes have been identified on the glycoproteins of other viruses, the next logical proteins of Pichinde to investigate for CTL epitopes on these haplotypes were the glycoproteins.

1.1 vvGPC

A vaccinia virus recombinant expressing full-length Pichinde glycoprotein precursor was recently constructed in the laboratory by E. Wanas (1993). Briefly, this was done by first cloning GPC using standard procedures into a plasmid which contains some vaccinia virus sequence and then introducing the gene into wild-type vaccinia virus by homologous recombination in transfected cells. Experiments done using this recombinant, vvGPC, showed that Pichinde GPC could be immunoprecipitated from cells infected with the recombinant virus and that the recombinant could elicit

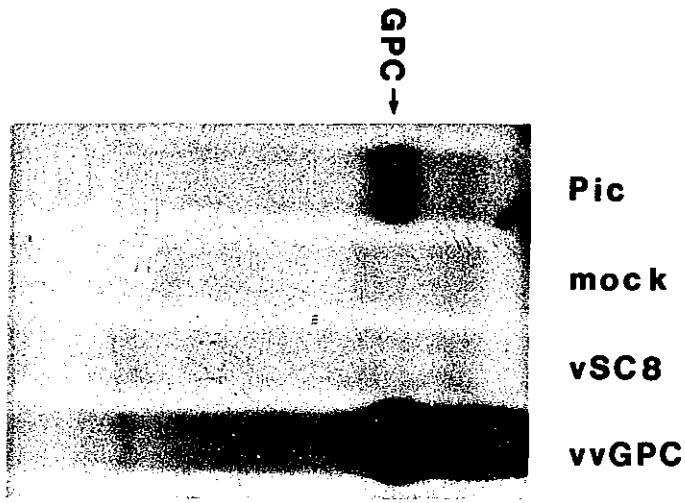
antibodies in rabbits that recognized Pichinde GPC in Western blots and in immunoprecipitation experiments (Wanas, E., 1993).

Although Pic-GPC was successfully immunoprecipitated from vvGPC infected BHK and CV-1 cells, it was decided to repeat the immunoprecipitation in the same cells and under the same infection conditions that would be used for the target cells in the CTL assays. 25 cm^2 flasks of MC57 cells were infected with Pichinde virus at a MOI of 1 for 24 hrs and with vSC8 (control vaccinia virus) and vvGPC at a MOI of 3 for 6 hrs. The proteins of the cells were labelled with ^{35}S-methionine and the viral glycoprotein immunoprecipitated with a monoclonal antibody specific for an epitope on GP-2. For accurate comparison, the amount of cell lysate used for all flasks was equivalent in cell number to one flask of Pichinde-infected cells. Figure 1 shows the results of this immunoprecipitation. As can be seen, the glycoprotein precursor of Pichinde is readily expressed in MC57 cells infected with either Pichinde or the recombinant virus, but not in cells infected with the control virus.

1.2 CTL assays with vvGPC

Chromium release assays were performed to determine whether CTL epitopes could be found on GPC. This was investigated 2 ways. First, if GPC contains an epitope, CTL derived against Pichinde virus should recognize and lyse vvGPC-infected cells. Second, if GPC contains an epitope then CTL derived against vvGPC should include GPC-specific CTL and therefore lyse Pichinde-infected targets as well as the vaccinia infected targets. Three separate experiments will be presented for each aspect

Figure 1: Immunoprecipitation of GPC from Pichinde- and vvGPC-infected MC57 cells. Cells were infected with Pichinde at a MOI of 1 for 24 hrs and with vSC8 and vvGPC at a MOI of 3 for 6 hrs. The cell lysates were labelled with ^{35}S-methionine and immunoprecipitated with a monoclonal antibody specific for GP-2. Each lanes represents an equivalent number of cells.


GPC
Pic
mock
vSC8
vvGPC

of this project since it is generally accepted that if the same results are found in 3 independent and valid CTL assays then conclusions can be drawn regarding the presence or absence of an epitope.

In order to ensure that any lysis observed is a result of CTL activity against the target, appropriate controls were included in every experiment. These controls consisted of mock infected cells (negative control), cells infected with another virus (virus control) and MHC-mismatched target cells infected with the homologous virus (MHC control). For Pichinde-specific effectors, targets were infected with Pic (positive control), vvGPC (test) and vSC8 (virus control); for vvGPC effectors, targets were infected with Pic (test), vvGPC and vSC8 (positive controls). To ensure that lack of lysis was not a result of lack of infection, Pic-specific effectors and vvGPC-specific effectors were generally considered in the same experiments.

The effectors used in these assays are secondary CTL. To derive these, mice are immunized with $2x10^7$ pfu of virus and left for at least three weeks. The spleens were then removed and the splenocytes restimulated *in vitro* for 5 days with infected syngeneic splenocytes. This secondary stimulation is necessary for many viruses in which the primary response is not reliably detectable in the chromium release assay. Although primary CTL are consistently used in assays with LCMV, numerous attempts to do so with Pichinde proved fruitless and therefore secondary CTL were generated and used instead.

Figures 2 and 3 show the results of one CTL assay done on the H-2^b murine haplotype. Effector cells were derived by immunizing mice with either Pichinde or

Figure 2: Lysis of target cells by Pic-specific CTL (H-2^b). Percent chromium released from infected MC57 (H-2^b) and Balb/c (H-2^d) target cells by effector cells derived against Pichinde virus. Cells were infected as follows: Pic and H-2^d Pic, MOI=1, 24 hrs; vSC8 and vvGPC, MOI=3, 6 hrs. 90% confidence intervals are shown for the positive control and mock-infected targets. The maximum intervals for the other targets are: vSC8, ±2.5; vvGPC, ±3; H-2^d Pic, ±5.5.

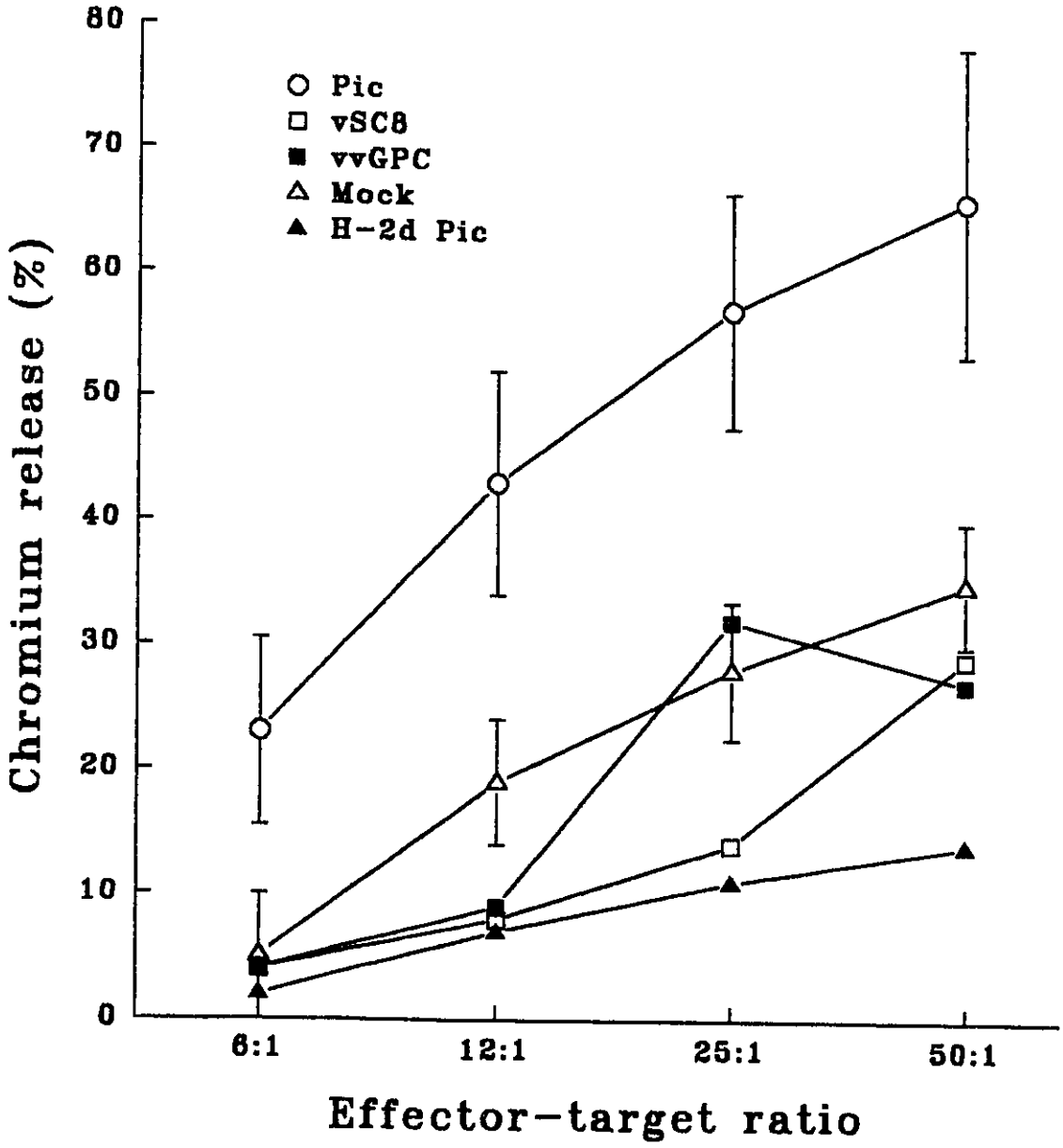
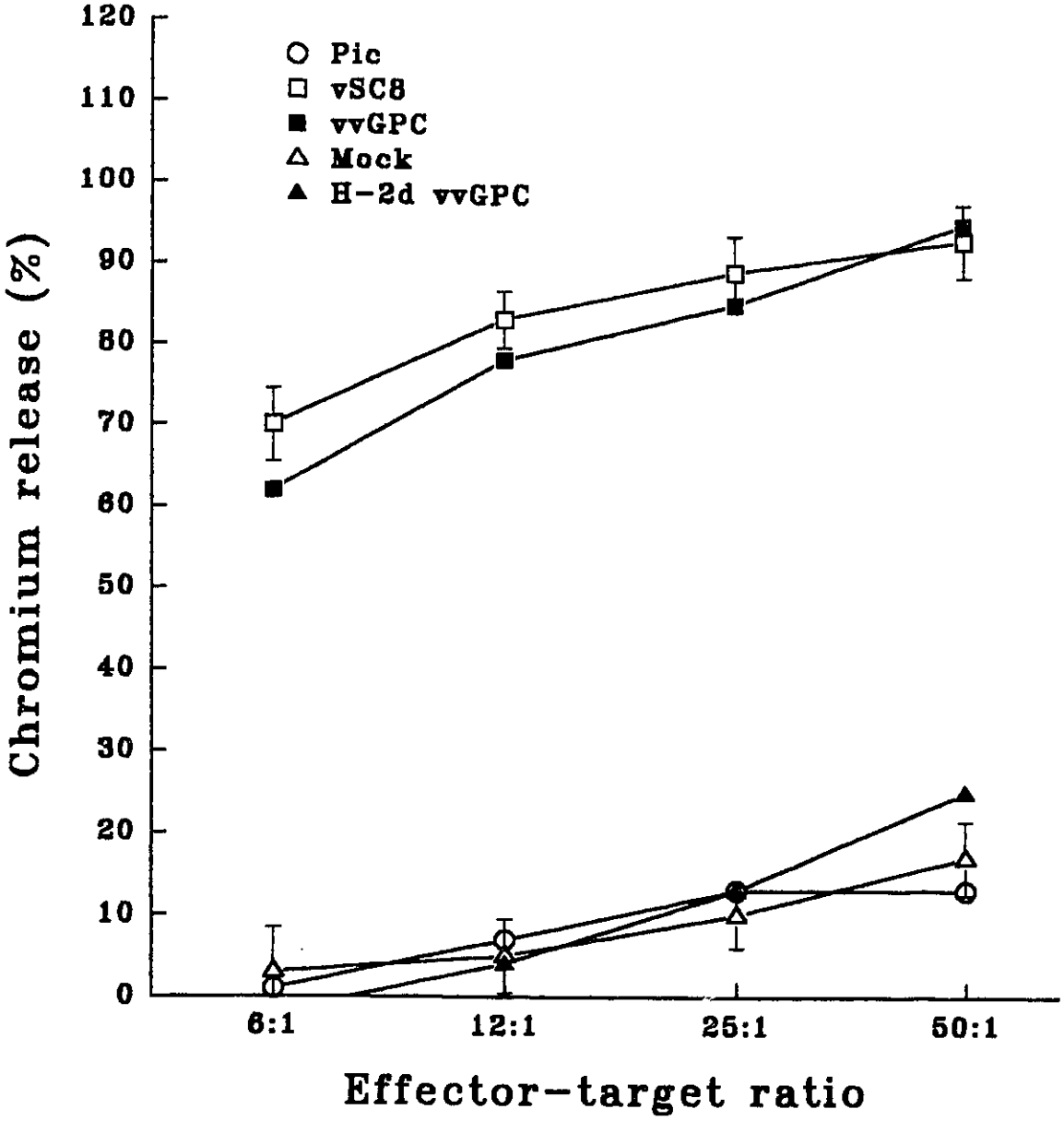

80
70
60
50
40
30
20
10
0
Chromium release (%)
Pic
vSC8
vvGPC
Mock
H-2d Pic
6:1
12:1
25:1
50:1
Effector-target ratio

Figure 3: Lysis of target cells by vvGPC-specific CTL (H-2^b). Percent chromium released from infected MC57 (H-2^b) and Balb/c (H-2^d) target cells by effector cells derived against vvGPC. Infection conditions are described in the legend of figure 2. 90% confidence intervals are shown for the positive controls and mock-infected targets. The maximum intervals for the other targets are: Pic, ±10; vvGPC ±4.5; H-2^d vvGPC, ±11.


Pic
vSC8
vvGPC
Mock
H-2d vvGPC
Chromium release (%)
120
110
100
90
80
70
60
50
40
30
20
10
0
6:1
12:1
25:1
50:1
Effector-target ratio

vvGPC and, after at least three weeks, restimulating the memory splenocytes with the same virus *in vitro* for 5 days. The resulting effector cells were then incubated with chromium labelled, infected MC57 (H-2^b, syngeneic) and Balb/c (H-2^d, allogeneic) cells. The graphs show the percent chromium release, calculated according to the formula described earlier, of the target cells by Pichinde-specific (Fig. 2) and vvGPC-specific (Fig. 3) effectors. The error bars represent the 90% confidence intervals which were determined according to the statistical equations derived in the appendix. To avoid confusion, these intervals are only presented on the graphs for the positive and negative controls. The ranges for the remaining targets are given in the legends. As can be seen, Pichinde-infected MC57 cells are readily lysed by Pichinde-specific CTL whereas vSC8, mock and the allogeneic control, Pichinde-infected H-2^d cells, show only a background level of lysis. Of note is that vvGPC-infected cells are lysed at the background level equivalent to the cells infected with the control recombinant, vSC8. In Figure 3, both the vaccinia recombinants are lysed by the vvGPC-specific effectors although Pichinde-infected cells are not. Again, the MHC-restriction of the CTL can be seen in the fact that these effectors do not lyse an allogeneic cell line infected with the vaccinia virus recombinant. The results of this experiment indicate that there are no CTL epitopes on the glycoproteins since Pichinde effectors did not recognize vvGPC-infected targets above background levels nor did vvGPC effectors recognize Pichinde-infected targets.

Tables 1 and 2 show the results of two other experiments performed on the H-2^b haplotype. These experiments were done in a similar manner as above using C57Bl/6

Table 1: Lysis of targets by Pic-specific and vvGPC-specific CTL (H-2^b) - Second assay. Percent chromium released from infected MC57 (H-2^b) and Balb/c (H-2^d) cells by effectors derived against Pichinde and vvGPC.

		TARGETS[1,2]					
		MC57					Balb/c
EFFECTORS	E:T	Pic-5	Pic-1	vSC8	vvGPC	Mock	Pic
Pichinde	50:1	40	45	19	22	20	3
(H-2^b)	25:1	33	39	12	16	-6	-1
	12:1	21	31	7	8	-15	-2
	6:1	5	15	5	5	-17	-3
vvGPC	50:1	2		78	74	18	
(H-2^b)	25:1	0		75	58	5	
	12:1	-4		62	52	6	
	6:1	-6		51	40	-16	

[1] Infection conditions: MC57 Pic-5 were infected at a MOI of 5 for 48 hrs, MC57 Pic-1 and Balb/c Pic were infected at a MOI of 1 for 48 hrs. The vaccinia recombinants, vSC8 and vvGPC, were infected at a MOI of 3 for 6 hrs.

[2] 90% confidence intervals: MC57 - Pic-5, ±18.5; Pic-1, ±23; vSC8, ±7; vvGPC, ±7.5 (Pic CTL), ±12.5 (vvGPC CTL); Mock, ±26; Balb/c - Pic, ±8.

Table 2: Lysis of targets by Pic-specific and vvGPC-specific CTL (H-2^b) - Third assay. Percent chromium released from infected MC57 (H-2^b) and Balb/c (H-2^d) cells by effectors derived against Pichinde and vvGPC.

		TARGETS[1,2]				
		MC57				Balb/c
EFFECTORS	E:T	Pic-5	Pic-1	vSC8	vvGPC	Pic
Pichinde	50:1	82	91	18	18	21
(H-2^b)	25:1	78	78	15	20	6
	12:1	59	73	5	13	5
	6:1	24	40	5	5	-4
vvGPC	50:1	2		75	95	
(H-2^b)	25:1	-10		75	88	
	12:1	-9		77	80	
	6:1	-30		64	76	
vvGPC (unstim.)	50:1					
(H-2^b)	25:1			11	12	
	12:1			3	9	
	6:1			-1	1	

[1] Infection conditions: MC57 Pic-5 were infected at a MOI of 5 for 48 hrs while MC57 Pic-1 and Balb/c Pic cells were infected at a MOI of 1 for 48 hrs. The vaccinia targets were infected at a MOI of 3 for 6 hrs.

[2] 90% confidence intervals: MC57 - Pic-5, ±26; Pic-1, ±30; vSC8, ±9.5 (Pic CTL and unstimulated CTL), ±13.5 (vvGPC CTL); vvGPC, ±7 (Pic CTL and unstimulated CTL), ±11 (vvGPC CTL); Balb/c - Pic, ±10.5.

mice to derive the effectors and MC57 cells as the syngeneic target cell line. Infection conditions and values for the 90% confidence intervals are given in the table legends. Since the ranges for the confidence intervals for all data points for a given target (4 points/effector) are usually very similar (probably accounting for the fact that the same maximum and spontaneous values are used in the equations), only one value for the confidence intervals for that target will be stated unless otherwise noted. This value will represent the largest range of all that were calculated for the target and therefore it can be assumed that the ranges for all points are close to and either equal to or less than the value given. The results of both these experiments show that there is no significant difference in the recognition of vvGPC and the control recombinant, vSC8, by Pichinde-specific CTL. Also, Pichinde-infected targets are not recognized by vvGPC effectors. Table 2 also includes the data for the lytic activity of unstimulated vvGPC memory CTL on targets. As can be seen, these cells have no activity against the targets.

Overall, it appears that on the murine haplotype H-2^b there are no CTL epitopes on the glycoproteins of Pichinde virus.

Because the peptides that bind to an MHC class I glycoprotein are dependent on that molecule, CTL epitopes are specific for particular MHC alleles. For this reason, information that there are no CTL epitopes on one haplotype says little with respect to the presence or absence of such epitopes on another haplotype especially if they share no class I molecules. Separate experiments with the vaccinia recombinant were therefore

performed to determine whether there are any CTL epitopes on the Pichinde glycoproteins on the H-2^d murine background.

Figures 4 and 5 illustrate the lysis of target cells by Pic-specific and vvGPC-specific effectors, respectively. Tables 3 and 4 are the results of another 2 assays done on this background. For these experiments effectors were derived by immunizing Balb/c mice with Pichinde and vvGPC and restimulating the splenocytes with the same virus *in vitro* as before. Target cells were infected or uninfected Balb/c cells (syngeneic) or MC57 or L929 cells (allogeneic).

As can be seen from the figures and tables, the lytic activity observed shows the MHC and virus restriction characteristic of CTL. In all cases the effectors derived against Pichinde recognized and lysed the Pic-infected syngeneic cell line but the negative controls, the Pic-infected allogeneic cell line, the mock infected targets and the targets infected with vSC8, were not lysed past background levels. The vaccinia targets were lysed by the vaccinia effectors thus confirming the infection of these cells. Figure 5, vvGPC effectors with various targets, also shows the lytic activity of unstimulated vvGPC memory cells on the targets. As before, these results demonstrate the importance of the secondary stimulation as the unstimulated cells showed no activity against the targets whereas the stimulated cells did.

Pertaining to the CTL epitopes on the glycoproteins, it can be seen that the lysis of vvGPC-infected cells by Pic-specific CTL is not significantly different than the lysis of the control vaccinia virus, vSC8, indicating that vvGPC is not recognized by these CTL any more than is vSC8. Also, Pic-infected Balb/c cells are not recognized by

Figure 4: Lysis of target cells by Pic-specific CTL (H-2^d). Percent chromium released from Balb/c (H-2^d) and MC57 (H-2^b) target cells by effector cells derived against Pichinde virus. Cells were infected as follows: Pic, MOI=1 and 5, 24 hrs; H-2^b Pic, MOI=1, 24 hrs; vSC8 and vvGPC, MOI=5, 6 hrs. 90% confidence intervals are shown for the positive controls and mock-infected targets. The maximum intervals for the other targets are: Pic (MOI=1), ±6; vSC8, ±4.5; vvGPC, ±9.5; H-2^b Pic, ±14.

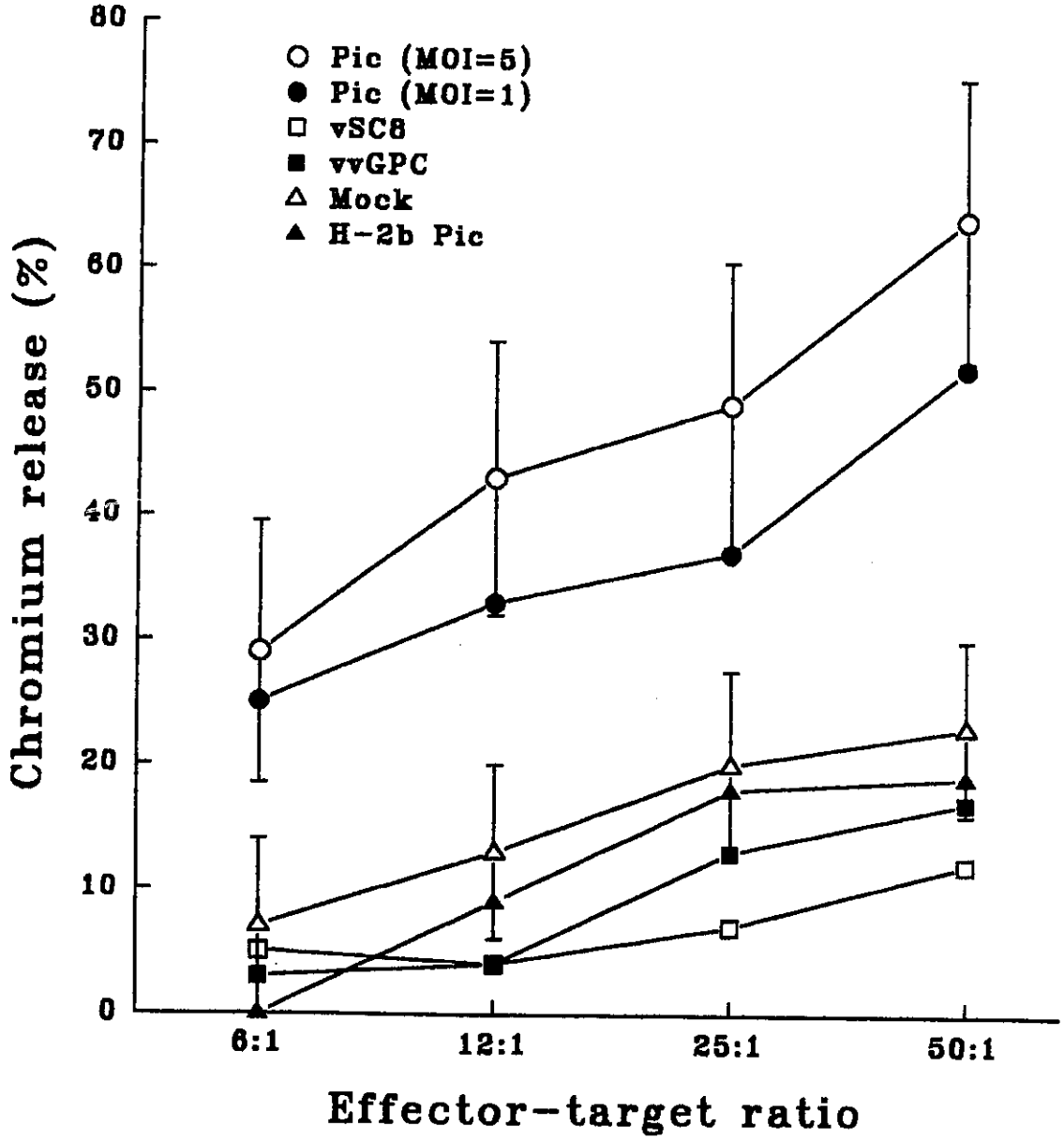

80
70
60
50
40
30
20
10
0
Chromium release (%)
Pic (MOI=5)
Pic (MOI=1)
vSC8
vvGPC
Mock
H-2b Pic
6:1
12:1
25:1
50:1
Effector-target ratio

Figure 5: Lysis of target cells by vvGPC-specific CTL (H-2^d). Percent chromium released from Balb/c (H-2^d) and MC57 (H-2^b) target cells by effector cells derived against vvGPC. Dashed lines represent lysis of targets by unstimulated effectors. Infection conditions are described in the legend of figure 4. 90% confidence intervals are shown for the positive controls and mock-infected targets. The maximum intervals for the other targets are: Pic (MOI=5), ±10.5; vvGPC, ±10.

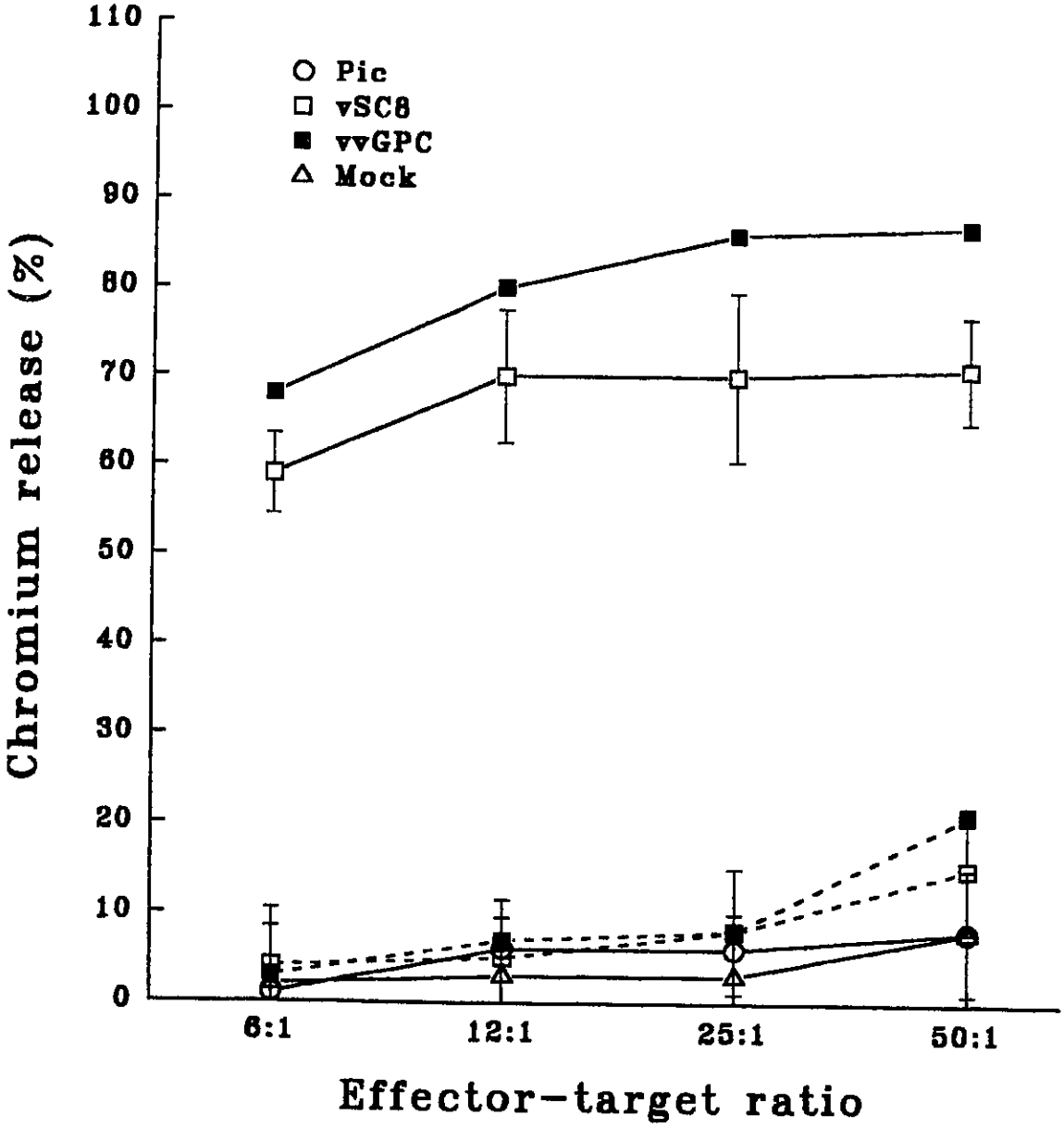

110
100
90
80
70
60
50
40
30
20
10
0
Chromium release (%)
Pic
vSC8
vvGPC
Mock
6:1
12:1
25:1
50:1
Effector-target ratio

Table 3: Lysis of targets by Pic-specific and vvGPC-specific CTL (H-2^d) - Second assay. Percent chromium released from infected Balb/c (H-2^d) and MC57 (H-2^b) cells by effectors derived against Pichinde and vvGPC.

		TARGETS[1,2]				
		Balb/c				MC57
EFFECTORS	E:T	Pic	vSC8	vvGPC	Mock	Pic
Pichinde	50:1	82	41	35	16	15
(H-2^d)	25:1	50	35	25	10	12
	12:1	46	21	18	4	6
	6:1	25	12	10	0	0
vvGPC	50:1	13	69	53	1	
(H-2^d)	25:1	9	73	47	2	
	12:1	12	61	41	0	
	6:1	6	57	37		

[1] Infection conditions: The Pichinde-infected targets were infected at a MOI of 10 for 48 hrs. The vaccinia targets were infected at a MOI of 1 for 12 hrs.

[2] 90% confidence intervals: Balb/c - Pic, ±10; vSC8, ±6 (Pic CTL), ±10 (vvGPC CTL); vvGPC, ±5; Mock, ±7; MC57 - Pic, ±10.5.

Table 4: Lysis of targets by Pic-specific and vvGPC-specific CTL (H-2^d) - Third assay. Percent chromium released from infected Balb/c (H-2^d) and L929 (H-2^k) cells by effectors derived against Pichinde and vvGPC.

		TARGETS[1,2]				
		Balb/c				L929
EFFECTORS	E:T	Pic	vSC8	vvGPC	Mock	Pic
Pichinde	50:1	70	37	30	24	18
(H-2^d)	25:1	65	32	25	10	15
	12:1	37	22	8	11	12
	6:1	38	11	12	0	5
vvGPC	50:1	23	50	65	21	
(H-2^d)	25:1	16	51	71	18	
	12:1	9	46	83	12	
	6:1	6	35	74	0	

[1] Infection conditions: The Pichinde targets were infected at a MOI of 5 for 48 hrs. The vaccinia targets were infected at a MOI=1 for 12 hrs.

[2] 90% confidence intervals: Balb/c - Pic, ±11; vSC8, ±15, vvGPC, ±18.5; Mock, ±12; L929 - Pic, ±9.

vvGPC-specific effectors. These facts indicate that no GPC-specific CTL are elicited by Pichinde or vvGPC that would recognize vvGPC or Pic-infected cells. This suggests that on the H-2^d background, like on the H-2^b background, there are no CTL epitopes on the glycoproteins of Pichinde virus.

2. TS MUTANTS

Temperature sensitive mutants of Pichinde have been derived (Vezza and Bishop, 1977; Shivaprakash et al., 1988). Two of the mutants, TS13 and TS908, were shown to be defective in the processing of the glycoproteins. At the non-permissive temperature, 39.5^{0}C, the precursor is not cleaved into its components, GP-1 and GP-2, although this cleavage does occur at 34^{0}C. The TS phenotype was mapped to the S genome segment although the mutants were derived by chemical mutagenesis and therefore changes are expected throughout the viral genomes. It was of interest to compare the CTL epitopes of the wild-type and TS viruses to see if the epitopes have been maintained or if they have been disrupted.

CTL assays were performed in mice of the same haplotypes considered previously. Effectors against Pichinde were derived as before by immunizing mice (C57Bl/6 and Balb/c) and restimulating the memory splenocytes with Pichinde *in vitro*. Target cells were MC57 and Balb/c cell lines infected with wild-type Pichinde virus or the TS mutants. The TS mutant targets were kept at 34^{0}C during the infection period. The CTL assays were done as before by incubating the effectors with chromium-

labelled target cells. The figures and tables show the percent chromium released from the various targets.

Figure 6 and Tables 5 and 6 show the results of three assays on the H-2^b murine haplotype. The graph and the tables show the percent chromium released from the labelled target cells by Pichinde-specific effectors. As can be seen, targets infected with TS13 appear to be lysed at equivalent levels to wild-type virus in all three of the experiments. Cells infected with TS908, on the other hand, lyses at a level comparable to or less than the virus control, vSC8, in two of the experiments (Fig. 6 and Table 6) and slightly greater than vSC8 in the third experiment (Table 5). This raises some questions as to whether TS908 may be recognized inefficiently by the effectors. It is definitely the case, though, that it is not recognized to the same degree as the wild-type or TS13 viruses.

Figure 7 and Tables 7 and 8 show the percent chromium released from targets in three assays on the H-2^d murine haplotype. For the three assays on this haplotype, neither targets infected with TS13 nor TS908 appear to be recognized by the Pic-specific effectors although wild-type virus is.

Therefore, it appears that the CTL epitopes recognized on the H-2^b haplotype are similar between TS13 and wild-type Pichinde viruses but that these epitopes are altered in TS908. On the H-2^d haplotype, neither TS mutant appears to share the wild-type virus' epitopes.

Ideally it would have been desirable to derive CTL against the two TS mutant viruses. Matching these effectors with the TS-infected targets would have confirmed the

Figure 6: Lysis of Pichinde wild-type and TS targets by Pic-specific CTL (H-2^b). Percent chromium released from MC57 (H-2^b) cells by effector cells derived against Pichinde. The wild-type and TS targets were infected at a MOI of 5 for 48 hrs, while the vSC8 targets were infected for 12 hrs at a MOI of 1. 90% confidence intervals are shown for the positive control. The maximum intervals for the other targets are: TS13, ±8; TS908, ±6.5; vSC8, ±11; H-2^k - Pic, ±4.

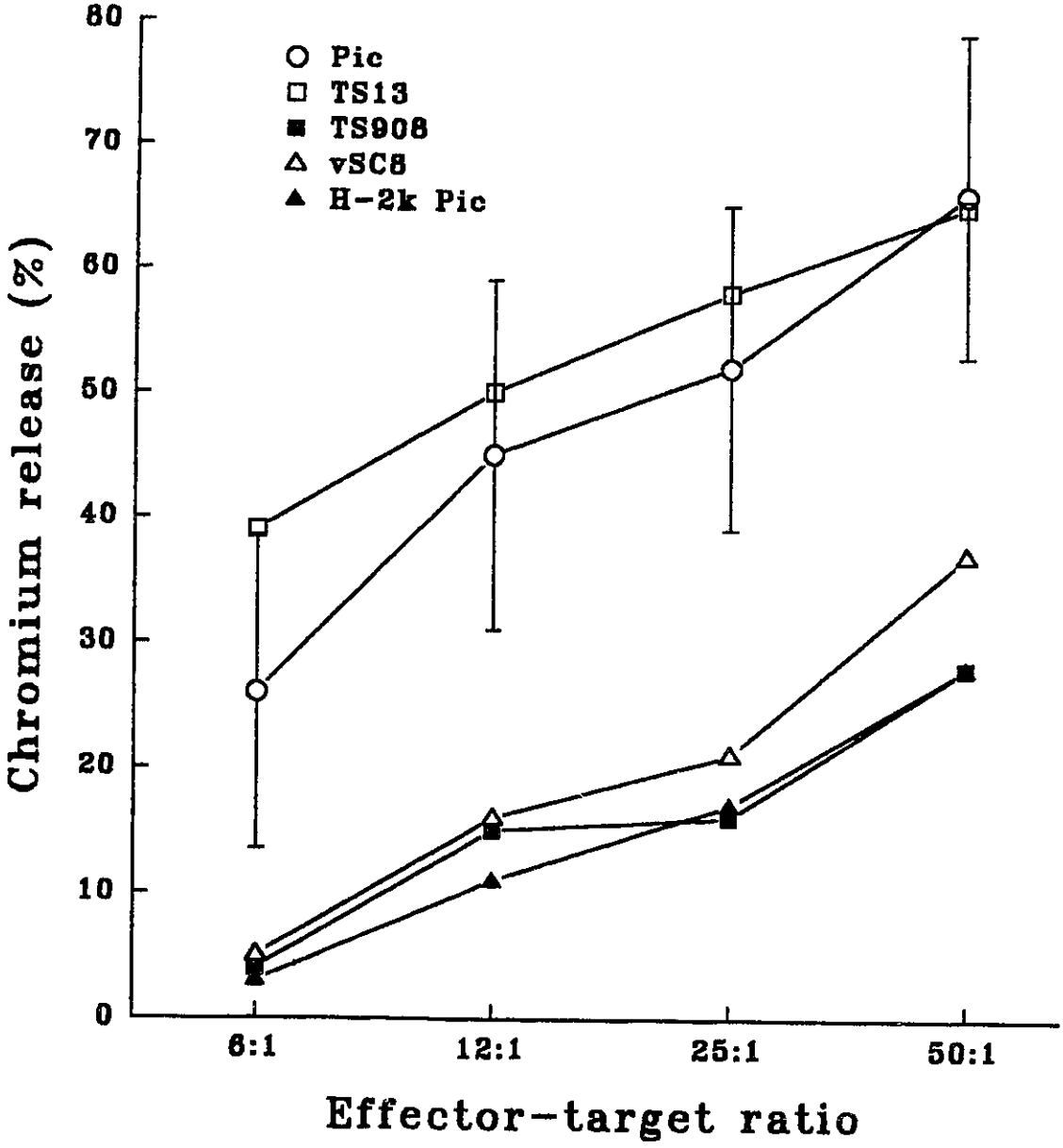

Pic
TS13
TS908
vSC8
H-2k Pic
Chromium release (%)
0
10
20
30
40
50
60
70
80
6:1
12:1
25:1
50:1
Effector-target ratio

Table 5: Lysis of Pichinde wild-type and TS targets by Pic-specific CTL (H-2^b) - Second assay. Percent chromium released from infected MC57 (H-2^b) targets by effectors derived against Pichinde.

		TARGETS[1,2]				
		MC57				
EFFECTORS	E:T	Pic	TS13	TS908	vSC8	Mock
Pichinde	50:1	71	58	42	23	16
(H-2^b)	25:1	47	47	34	14	14
	12:1	50	36	27	7	9
	6:1	28	22	21	2	5

[1] Infection conditions: The wild-type and TS targets were infected at a MOI of 1 for 48 hrs. The vaccinia target was infected at a MOI of 3 for 6 hrs.
[2] 90% confidence intervals: Pic - ±11.5; TS13, ±8; TS908, ±5; vSC8, ±5.5; Mock, ±15.

Table 6: Lysis of Pichinde wild-type and TS targets by Pic-specific CTL (H-2^b) - Third assay. Percent chromium released from infected MC57 (H-2^b) target cells by effectors derived against Pichinde.

		TARGETS[1,2]				
		MC57				L929
EFFECTORS	E:T	Pic	TS13	TS908	vSC8	Pic
Pichinde	50:1	52	60	22	31	30
(H-2^b)	25:1	48	46	21	34	18
	12:1	32	34	9	15	6
	6:1	21	20	4	0	0

[1] Infection conditions: The wild-type and TS viruses were infected at a MOI of 5 for 24 hrs. The vaccinia target was infected for 14 hrs at a MOI of 1.
[2] 90% confidence intervals: Pic, ±8.5; TS13, ±9; TS908, ±8; vSC8, ±15, L929 - Pic, ±5.

Figure 7: Lysis of Pichinde wild-type and TS targets by Pic-specific CTL (H-2^d). Percent chromium released from infected Balb/c (H-2^d) and MC57 (H-2^b) cells by effectors derived against Pichinde. Pichinde wild-type virus was used to infect the Balb/c cells at a MOI of 10 for 48 hrs and the MC57 cells at a MOI of 5 for 48 hrs. The TS mutants targets were also infected at a MOI of 5 for 48 hrs. 90% confidence intervals are shown for the positive control. The maximum intervals for the other targets are: TS13, ±13; TS908, ±6.5; mock, ±9; H-2^b Pic, ±12.5.

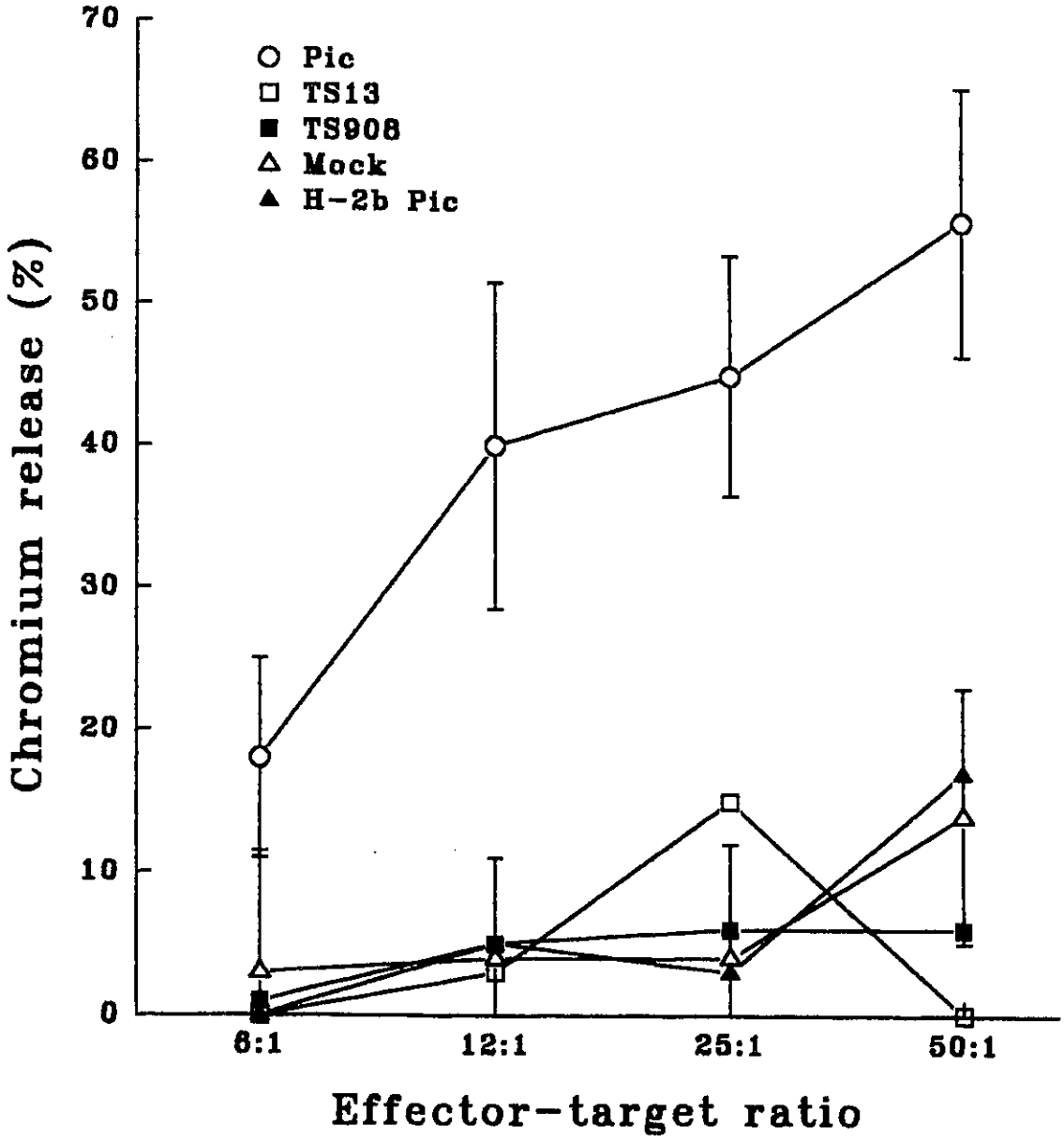

70
60
50
40
30
20
10
0
Chromium release (%)
Pic
TS13
TS908
Mock
H-2b Pic
6:1
12:1
25:1
50:1
Effector-target ratio

Table 7: Lysis of Pichinde wild-type and TS targets by Pic-specific CTL (H-2^d) - Second assay. Percent chromium released from infected Balb/c and MC57 targets by effectors derived against Pichinde.

		TARGETS[1,2]				
		Balb/c				MC57
EFFECTORS	E:T	Pic	TS13	TS908	Mock	Pic
Pichinde	50:1	55	2	18	13	28
(H-2^d)	25:1	40	5	11	8	19
	12:1	32	5	7	4	16
	6:1	21	-2	5	2	10
Pichinde	50:1	19				63
(H-2^b)	25:1	15				59
	12:1	11				45
	6:1	11				32

[1] Infection conditions: The Pichinde wild-type and TS targets were infected at a MOI of 1 for 48 hrs.
[2] 90% confidence intervals: Balb/c - Pic, ±7; TS13, ±19; TS908, ±15; Mock, ±8.5; MC57 - Pic, ±14.5.

Table 8: Lysis of Pichinde wild-type and TS targets by Pic-specific CTL (H-2^d) - Third assay. Percent chromium released from infected Balb/c target cells by effectors derived against Pichinde.

		TARGETS[1,2]			
		Balb/c			
EFFECTORS	E:T	Pic	TS13	TS908	Mock
Pichinde	50:1	73	31	21	25
(H-2^d)	25:1	80	30	21	26
	12:1	65	24	15	16
	6:1	46	22	12	6

[1] Infection conditions: Pic and TS, MOI=5, 24 hrs.
[2] 90% confidence intervals: Pic, ±16.5 (except 25:1, ±22); TS13, ±10; TS908, ±13.5; Mock, ±13.5.

infection of these targets and it would have allowed the determination of any reciprocal cross-reactivity between the TS and wild-type viruses. Unfortunately, numerous attempts to generate TS-specific CTL failed. There were several possible reasons for this, one of which was that these temperature sensitive viruses might not replicate adequately to generate a primary CTL response in the mice. Also, even if a CTL response was generated, there is the question of possible reversion of the TS mutants back to wild-type virus when these viruses replicated in mice. This would lead to doubts regarding the actual specificity of the CTL that would be generated. In an attempt to address these questions an infectious centres assay was performed.

The infectious centres assay measures the number of cells producing virus in the spleens of infected mice. Three Balb/c mice were infected with each of the three viruses. The mice were sacrificed 2, 3 and 4 days after immunization. The spleens were removed, ground up and diluted to a concentration of 10^6 cells/mL. Dilutions of the cell preparations were then used to infect Vero cells as in a plaque assay. An overlay was added to the cells after a 1 hour absorption period and a second overlay with neutral red added 4 days later. The plates with wild-type virus were incubated at 37^0C while one set of plates for each of the TS mutants was incubated at 34^0C and another set at 39^0C. Plaques were counted the day after the second overlay was added.

Table 9 shows the number of infectious centres per 10^6 splenocytes in mice infected 2, 3 or 4 days with wild-type, TS13, or TS908. As can be seen from the results, wild-type virus and TS908 appear to replicate in the mice while TS13 does not. Also, the virus in the spleens of TS908 infected mice has retained its TS phenotype

Table 9: Infectious centres assay. Numbers of infectious centres per 10^6 cells in the spleens of mice infected for 2, 3 or 4 days with Pichinde wild-type or TS viruses.

LENGTH OF INFECTION (DAYS)	Pic	TS13		TS908	
	37°C	34°C	39°C	34°C	39°C
2	408	5	0	1617	0
3	465	27	0	3150	0
4	93	0	0	992	0

over the 4 days of infection as indicated by the lack of plaques at the non-permissive temperature.

DISCUSSION

1. CTL epitopes of Pichinde virus

This report presents data showing that the glycoproteins of Pichinde virus do not contain CTL epitopes on two murine MHC backgrounds, H-2^b and H-2^d. This was determined using a vaccinia virus recombinant expressing the Pichinde glycoprotein precursor as a target antigen or eliciting antigen in the standard chromium release CTL assay. These two haplotypes were considered because no CTL epitopes were found on the nucleoprotein of this virus on these haplotypes. The glycoproteins were deemed the next logical proteins to examine for the epitopes responsible for CTL induction and recognition. The two haplotypes were considered independently because CTL epitopes are restricted to a given MHC class I molecule and are not generally shared between the different MHC molecules found in different inbred strains of mice. It may very well be that on other murine haplotypes, there are CTL epitopes on the glycoproteins or on the nucleoprotein.

The conclusion that there are no epitopes on the glycoproteins was arrived at because the recombinant expressing the glycoprotein precursor was not able to induce anti-Pichinde CTL in mice nor could it sensitize target cells for lysis by CTL specific for Pichinde. This was observed on both the murine backgrounds studied. Numerous investigations into the CTL epitopes of many viruses including LCMV, influenza, and RSV have shown that if a recombinant vaccinia virus expresses a protein which contains a CTL epitope then antiviral CTL would be generated in mice that recognize virus-infected cells and that cells infected with the recombinant would be recognized and killed by CTL generated against whole virus. (Whitton et al., 1993; Bennink et al.,

1987; Bangham et al., 1986). Since the recombinant vaccinia virus is efficient at expressing GPC (Fig. 1), the lack of lysis observed in these experiments is most likely a result of the absence of CTL epitopes.

The conclusion that Pichinde GPC does not have CTL epitopes on H-2^b or H-2^d is not in itself unusual. Lymphocytic choriomeningitis virus (LCMV), another arenavirus, was also found not to have such epitopes on the glycoproteins on several murine haplotypes examined. The results of Whitton et al. (1988a) and Hany et al. (1989) showed that only on 2 out of 6 murine haplotypes was there a response directed against the glycoproteins. In contrast, on 4 or 5 of these backgrounds there was a response against the LCMV nucleoprotein (Hany et al., 1989; Lewicki et al., 1992). Similarly, for respiratory syncytial virus (RSV) and vesicular stomatitis virus (VSV), the nucleoprotein but not the glycoprotein was recognized by human and murine CTL (Puddington et al., 1986; Bangham et al., 1986). What is unusual about Pichinde virus is that it does not have epitopes on either the glycoproteins or the nucleoprotein on these two haplotypes. No other virus has yet shown these results. This suggests, therefore, that the CTL epitopes of Pichinde virus on these MHC backgrounds are on the L protein and/or the putative Z protein. This latter protein has not yet been identified for Pichinde. It has been found associated with the nucleic acids of Tacaribe and LCMV and is coded on the 5' end of the L genome segment in these viruses.

Although the polymerase proteins of many viruses have not yet been examined for CTL epitopes, there are several reports where such epitopes have been found on these proteins. Bennink et al. (1987) showed that among three murine haplotypes all

three polymerases of influenza were recognized. These results were substantiated by Reay et al. (1988, 1989) who identified the regions of the epitopes on PB1 and PB2 on H-2^k and H-2^d and showed that these epitopes were responsible for a portion of the cross-reactive CTL in polyclonal anti-influenza CTL. Similar experiments by Shirai et al. (1992) showed that the RNA polymerase of hepatitis C virus contains CTL epitopes on H-2^d. Recently, Lewicki et al. (1992) examined three anti-LCMV CTL clones which caused immunopathology in infected H-2^k mice. One of these clones is believed to be directed against the polymerase as it recognized a product on the large genome segment that was not Z.

Besides the polymerase and the nucleocapsid proteins, anti-viral CTL have been shown to recognize other internal proteins which lends support to the notion that CTL epitopes may be present on the putative Z protein of Pichinde virus as well. For influenza virus, human CTL have recognized an epitope on the matrix protein while some murine CTL have recognized the non-structural protein, NS1 (Bednarek et al., 1991; Bennink et al., 1987). An H-2^d restricted epitope on the non-structural immediate-early protein, pp89, of murine cytomegalovirus (MCMV) has been identified (Volkmer et al., 1987). This is believed to be a major epitope of this virus on this haplotype. When expressed in a recombinant vaccinia virus and used to immunize H-2^d mice, CTL are generated which protect these mice from subsequent lethal MCMV infection (Jonjic et al., 1988). A similar protein in herpes simplex virus type I, ICP27, was identified as harbouring a major CTL epitope in H-2^d mice (Banks et al., 1993). Some other viruses in which epitopes have been identified on internal proteins other

than the polymerase and nucleoproteins are rabies, respiratory syncytial virus, and Dengue virus (Larson et al., 1991; Kulkarni et al., 1993; Cherrie et al., 1992; Rothman et al., 1993).

Between the two inbred strains of mice used in these experiments, a total of six MHC class I molecules were being studied (K^b, D^b, L^b molecules in H-2^b mice and K^d, D^d, L^d in H-2^d mice). Through the sequence analysis of peptides eluted from several of these MHC class I mice glycoproteins and the delineation of many viral CTL epitopes, sequence motifs for some of the murine class I molecules have been proposed (Rothbard and Taylor, 1988; Rötzschke and Falk, 1991). These consensus sequences are specific for a particular MHC molecule and are usually 9 amino acids long with 2 amino acids, generally near the ends of the peptide, responsible for binding to the MHC molecule. Recently, these sequence motifs have been used to predict CTL epitopes. For example, Cossins et al. (1993) used the K^k motif to successfully identify a CTL epitope on the NS1 protein of influenza on the H-2^k haplotype. With this in mind, the amino acid sequence Pichinde GPC was examined to see if it contained sequences that would fit the motifs of the four MHC molecules, present in H-2^b and H-2^d mice, for which such sequences have been described. These molecules are K^b and D^b in H-2^b mice and K^d and L^d in H-2^d mice (Rötzschke and Falk, 1991; Corr et al., 1992). This search revealed several possible MHC binding peptides for each allele. This would tend to suggest that perhaps GPC does contain an epitope despite the results presented here. To examine this possibility, a similar analysis was done with LCMV GPC whose epitopes have already been identified experimentally (Oldstone et al., 1988; Klavinskis et al.,

1990). This search revealed many sequences within GPC that satisfied the consensus sequences but which have not been found to be epitopes. As well, the D^b restricted epitopes of LCMV GPC previously determined were not predicted by this sequence analysis. This illustrates the unreliability of this method of predicting CTL epitopes at the present time. A deeper understanding of the roles of the various amino acids in and flanking the CTL epitope in antigen processing and presentation and in CTL recognition is needed before such epitopes can be accurately and regularly predicted from the amino acid sequence.

It is possible that there are CTL epitopes on the glycoproteins of Pichinde virus that are not being detected here. One way that this might happen is if CTL epitopes of vaccinia virus are outcompeting the Pichinde epitopes for the available MHC molecules. This could be due to a higher affinity of the vaccinia epitopes for the MHC. This phenomenon has not been reported to occur although techniques have not been available to test this possiblity. It should be pointed out, though, that CTL are believed to require only a very small number of MHC/peptide complexes to properly recognize their targets (Christinck et al., 1991). Given the amount of GPC produced in the infected cells, it is highly unlikely that the CTL epitopes would not be presented in sufficient quantities to activate the CTL and provoke CTL mediated lysis. With the advent of newer techniques such as sensitizing cells with protein by way of liposomes, the possiblity of competition among the epitopes would be avoided as the protein would be processed and presented in the absence of any potentially competing proteins (Reddy et al., 1991).

2. TS mutant results

The results presented here suggest that the H-2^b restricted epitopes of wild-type Pichinde are also present in the Pichinde temperature sensitive mutant, TS13, and that they appear to be disrupted in TS13 on the H-2^d haplotype and in another mutant, TS908, on both haplotypes. The disruptions may be a result of as little as a single amino acid substitution in the epitope or in the sequence surrounding the epitope. Such an amino acid change may affect the antigen processing (ex. proteolytic cleavage and/or peptide transport) or presentation of the peptide (ex. binding to the MHC) or it may alter the CTL recognition.

In the TS mutants, the TS phenotype has been mapped to the small genome segment. Neither mutant shows cleavage of the glycoprotein precursor at the non-permissive temperature (Shivaprakash et al., 1988). This is most likely a result of amino acid changes in the glycoprotein precursors of the mutants. It was expected and hoped that Pichinde CTL would recognize the TS-infected targets. Since there appears to be no epitopes on the glycoproteins, mutations in these proteins should not be disrupting CTL epitopes and therefore one might expect Pichinde CTL to recognize the TS targets. It is of no great cause for concern, though, that this is not the case. The mutants were derived by chemical mutagenesis which should result in changes throughout the genome. Although the glycoproteins may be more permissive to mutations, it is quite possible and likely that there are mutations in the other proteins as well. Mutations in the L (and Z) proteins may have resulted in the disruption of CTL epitopes present on

these proteins. Comparing the sequences of the genes of the wild-type and TS viruses may aid in the elucidation of these epitopes.

It would have been desirable to have generated TS-specific CTL in the experiments with these mutants. With these CTL, any cross-reactivity, such as between Pichinde and TS13 on the H-2^d background or between the TS mutants themselves, could have been examined. These CTL would have also confirmed the infection of the target cells although the consistent lysis of H-2^d targets infected with TS13 indicates that the infection of the cells is probably satisfactory. Unfortunately, attempts to generate such CTL by immunizing mice with the viruses and restimulating the resulting memory splenocytes *in vitro* failed. For some reason, the splenocytes in the flasks proliferated rapidly and died before the fifth day of culture. This effect was more noticeable for TS908 than TS13. Changing the media in the flasks, splitting the cells and starting with fewer cells did little to improve the cell survival at the end of the culture. It is believed that this excessive proliferation was caused by contamination of the virus stocks possibly with mycoplasma. The infection of the target cells was probably not affected by this, though. The consistent lysis of TS13 on the H-2^b haplotype, the background level of lysis in all other cases, and the similarity of the TS targets to the Pichinde targets in appearance and in the spontaneous and maximum values and the ratio between these, make it unlikely that anything abnormal is occurring in these targets.

There was a concern that the TS mutants may not be able to adequately invoke a CTL response in the mice. Also, it was possible that during replication in the mice,

the viruses may lose their TS phenotype and revert back to wild-type. The results of the infectious centres assay which measures virus producing cells in the spleen, indicated that TS908 but not TS13 replicates in mice. This may mean that TS13 will not be able to generate a CTL response as it may not be present in sufficient quantities for a long enough time to do so. The infectious centres assay also revealed that despite replicating in mice, TS908 did not lose its TS phenotype after 4 days of infection.

3. CTL Assay

The chromium release assay is a widely used technique to examine cytolysis by cells such as CTL and natural killer cells. This assay along with tools such as recombinant vaccinia viruses has furthered the understanding of CTL activity and allowed the elucidation of many viral CTL epitopes. In these experiments, this assay was used to examine the glycoproteins of Pichinde virus for CTL epitopes and to compare the CTL epitopes of Pichinde and the TS viruses. Several problems were encountered in doing this assay which will be discussed here.

First, there was often a high background level of lysis in the assays. This was more common with some targets such as mock infected Balb/c cells. Several experiments were discarded as a result of an excessively high background lysis of the control cells. Also, the vaccinia and TS experiments were performed several times on a third murine haplotype, H-2^k. Ozols et al. (1990) had shown that there were no CTL epitopes on the Pichinde nucleoprotein on this haplotype as well as on the other two. Unfortunately, despite many attempts, the background level of lysis for the mock- and

vSC8-infected L929 target cells was consistently too high to be able to accurately interpret the results. It is not known why this high background was occurring. It may be related to the batch of cells used although the same batch used at different times often gave varying levels of background lysis. It should be noted that, despite background, the positive controls were regularly lysed at higher levels.

Another problem frequently encountered was the difficulty in achieving a ratio between the maximum counts and spontaneous counts sufficiently high so as to ensure the accuracy of the chromium release values calculated. For CTL assays using the murine cells used here, this ratio is generally reported as being 3 or more for the various targets. In the experiments in this work the maximum to spontaneous ratio varied depending on the targets. The vaccinia targets infected at a MOI of 3 gave the best ratios which were often as high as 7, while the mock infected cells were the worst with the maximum values sometimes only two times the spontaneous values. The ratios for the Pichinde-infected targets were generally acceptable with average ratios between 2.5 and 3 for the MC57 cells and closer to 3.5 for the Balb/c cells. Low maximum to spontaneous ratios are the reason why the data for the mock-infected targets are not presented for some of the experiments in this work. This does not invalidate the experiment as the problem was strictly with the mock cells and not with the infected cells. It is not known why this problem arose only with some targets and not with others.

Another technique for the CTL assay has been proposed in order to overcome some of the disadvantages of the chromium release assay (Suhrbier et al., 1991). The

BLT assay is based on the principle that CTL release serine esterases when they come in contact with their target cells. These serine esterases are believed to play a role in target cell lysis. The level of the esterases in the supernatants of the cultures after the incubation of the effectors and targets can be measured using a colorimetric reaction with an esterase substrate, BLT. There are several advantages of this assay. It is non-radioactive and, because there is no concern of radioactive decay or of chromium leaking from the target cells, the assay can be left for longer and the plates stored and analyzed at a later date. It also has the advantage that it measures CTL activity directly and not indirectly by way of target cell lysis. This would alleviate the problems experienced here of high background and poor maximum to spontaneous ratios in the target cells.

In order to investigate this assay as a possibility, the BLT assay was performed in parallel to the chromium release assay. The results of this chromium release assay are shown in Figures 2 and 3. As can be seen, the radioactive assay worked quite well with the positive controls lysing well above the negative controls especially for the vaccinia effectors. The BLT assay did not show these results. The detergent, NP-40, released esterases from the CTL but there was no significant difference between the esterase released by the vaccinia effectors in response to the vaccinia targets as compared to the negative control targets as measured by this assay. It may be that the conditions for the BLT assay need to be optimized although Suhrbier et al. (1991) claimed that both assays worked equally well under the same conditions. Welsh et al. (1990), while examining the mechanism of killing of anti-LCMV CTL using the BLT

assay, found that very little of the total esterase is released from the CTL when these cells are incubated with infected target cells. In this report, the authors suggest that it may be difficult to detect esterase released from CTL specific for viruses that do not induce CTL as efficiently as LCMV.

4. Future work

Since there appear to be no CTL epitopes on the Pichinde virus glycoproteins or nucleoprotein on two murine haplotypes, it is of interest to examine L and Z for possible epitopes on these haplotypes. This can be done by cloning these genes and expressing them in vaccinia virus recombinant as in these experiments or by newer techniques such as introducing the proteins into cells by liposomes or pinocytosis. Cloning L and Z (if present) would have the added advantage of investigating these genes as no work has yet been done on the large genome segment of Pichinde.

Future work with the TS mutants may involve further attempts to generate TS-specific CTL and to observe the cross-reactivity between these and the wild-type virus. Sequencing studies of these viruses and comparisons to wild-type sequences may help identify any CTL epitopes as well as in investigating the TS defect of the mutants.

5. Conclusions

The glycoproteins, GP-1 and GP-2, of Pichinde virus do not contain any H-2^b- or H-2^d- restricted CTL epitopes. A vaccinia virus recombinant expressing the full-length glycoprotein precursor, GPC, of Pichinde was unable to induce CTL in mice that

recognized Pichinde-infected target cells nor could the recombinant sensitize target cells for lysis by Pichinde-specific CTL. These results imply that the CTL epitopes of Pichinde virus on the H-2^b and H-2^d murine haplotypes reside on the polymerase protein and/or the putative Z protein.

Comparisons of the CTL epitopes of Pichinde wild-type and two temperature sensitive mutants, TS13 and TS908, by examining the lysis of infected cells by Pic-specific CTL, showed that the wild-type virus and TS13 share the same CTL epitopes on H-2^b while the epitopes in TS908 are different from that of wild-type. On H-2^d, neither TS mutant appears to share the wild-type virus' epitopes.

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APPENDIX

The following is the mathematical derivation that was used to calculate the confidence intervals for the chromium release values.

x_1, x_2, x_3, x_4 = cpm for sample wells
b_1, b_2, b_3, b_4 = cpm for spontaneous release wells
m_1, m_2, m_3, m_4 = cpm for maximum release wells

$$\bar{x}=\frac{\sum_{i=1}^{4} x_i}{4} \qquad \bar{b}=\frac{\sum_{i=1}^{4} b_i}{4} \qquad \bar{m}=\frac{\sum_{i=1}^{4} m_i}{4}$$

Assumptions:

1. x_i, b_i, and m_i (i=1 to 4) are from populations X, B, and M which are normally distributed with means μ_x, μ_b, μ_m and variances $k_x\sigma^2$, $k_b\sigma^2$, and $k_m\sigma^2$ respectively.

2. X, B, M are independent.

3. The ratio of the variances of X, B, and M is k_x=9, k_b=1, and k_m=9. This implies that there is an underlying variance σ^2, set approximately equal to the variance in the spontaneous wells, which is increased as the counts increase. The maximum which is generally three times the spontaneous would therefore have nine times the variance of the spontaneous. The sample which generally falls between the spontaneous and the maximum will have a maximum variance equivalent to that of the maximum.

4. $\mu_m > \mu_b$

Purpose:

To derive equations that would enable the calculation of confidence intervals for the statistic:

$$\frac{\mu_x-\mu_b}{\mu_m-\mu_b}$$

Consider the statistic $\bar{x}-u\bar{m}-(1-u)\bar{b}$ where u is a real-valued variable.
This has a normal distribution since it is made up of the sums and differences of normal distributions.
The mean and variance of this statistic are:

$$mean=\mu_x-u\mu_m-(1-u)\,\mu_b \qquad variance=\frac{k_x\sigma^2}{4}+\frac{u^2k_m\sigma^2}{4}+\frac{(1-u)^2k_b\sigma^2}{4}$$

Therefore,

$$Z=\frac{[\bar{x}-u\bar{m}-(1-u)\,\bar{b}]-[\mu_x-u\mu_m-(1-u)\,\mu_b]}{\sigma\sqrt{\frac{k_x+u^2k_m+(1-u)^2k_b}{4}}}$$

has a standard normal distribution.

σ^2, the underlying variability in the chromium release values, is not known. An unbiased estimator for σ^2 can be obtained by pooling the data for x, b, and m:

$$S_p^2=\frac{\frac{1}{k_x}\sum_{i=1}^{4}(x_i-\bar{x})^2+\frac{1}{k_b}\sum_{i=1}^{4}(b_i-\bar{b})^2+\frac{1}{k_m}\sum_{i=1}^{4}(m_i-\bar{m})^2}{3+3+3}$$

Now,

$$\chi^2=\frac{9\,S_p^2}{\sigma^2}$$

has a chi-squared distribution with $\upsilon = 9$ degrees of freedom.
So,

$$T=\frac{Z}{\sqrt{\frac{\chi^2}{\nu}}}$$

has a t-distribution where $\upsilon = 9$ degrees of freedom.

Substituting the values for Z, χ^2, and υ gives:

$$T=\frac{[\bar{x}-u\bar{m}-(1-u)\bar{b}]-[\mu_x-u\mu_m-(1-u)\mu_b]}{S_p\sqrt{\frac{k_x+u^2k_m+(1-u)^2k_b}{4}}}$$

T has a t-distribution with 9 degrees of freedom.
Following the rules of t-distributions:

$$P(-t_{\frac{\alpha}{2}}<T<t_{\frac{\alpha}{2}})=1-\alpha$$

Substituting and rearranging the terms gives:

$$P(p<\mu_x-u\mu_m-(1-u)\mu_b<q)=1-\alpha$$

where

$$p=\bar{x}-u\bar{m}-(1-u)\bar{b}-t_{\frac{\alpha}{2}}S_p\sqrt{\frac{k_x+u^2k_m+(1-u)^2k_b}{4}} \qquad (1)$$

and

$$q=\bar{x}-u\bar{m}-(1-u)\bar{b}+t_{\frac{\alpha}{2}}S_p\sqrt{\frac{k_x+u^2k_m+(1-u)^2k_b}{4}} \qquad (2)$$

By the symmetry of the t-distribution:

$$P(p<\mu_x-u\mu_m-(1-u)\mu_b)=1-\frac{\alpha}{2} \qquad (3)$$

$$P(\mu_x-u\mu_m-(1-u)\mu_b<q)=1-\frac{\alpha}{2} \qquad (4)$$

Solve equation **1** for the value of u which gives p=0. Call this u_1.
Solve equation **2** for the value of u which gives q=0. Call this u_2.

From equations **3** and **4**:

$$P(0 < \mu_x - u_1\mu_m - (1-u_1)\mu_b) = 1 - \frac{\alpha}{2}$$

$$P(\mu_x - u_2\mu_m - (1-u_2)\mu_b < 0) = 1 - \frac{\alpha}{2}$$

That is,

$$P(u_1 < \frac{\mu_x - \mu_b}{\mu_m - \mu_b}) = 1 - \frac{\alpha}{2}$$

and

$$P(u_2 > \frac{\mu_x - \mu_b}{\mu_m - \mu_b}) = 1 - \frac{\alpha}{2}$$

So,

$$P(u_1 < \frac{\mu_x - \mu_b}{\mu_m - \mu_b} < u_2) = 1 - \alpha$$

which gives the desired confidence interval.