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T LYMPHOCYTE RESPONSES TO EQUID HERPESVIRUSES 1 AND 4 IN HORSES

Terry O'Neill

A thesis submitted in fulfilment of the requirements
of the
Open University
for the degree of Doctor of Philosophy

Centre for Preventive Medicine
Animal Health Trust

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Preface

The work reported in this dissertation was carried out in the laboratories of the Centre for Preventive Medicine of the Animal Health Trust. The studies represent work carried out by Terry O'Neill. Where assistance was given due acknowledgement is given in the text. This dissertation has not been submitted as whole or in part to any other university.

A fold out list of abbreviations is situated at the end of this dissertation.

T. O'Neill 7/9/95.

Acknowledgements

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I would like to thank Dr. M. Whelan from the BBSRC Institute for Animal Health, Compton for performing the IEF typing of the horses used in chapter 5 and for valuable discussions. I am also grateful to Dr. M. Wills from the Department of Medicine, University of Cambridge who advised me on the use of the split well LDA.

All of the laboratory, secretarial and estate staff at the Animal Health Trust, Centre for Preventive Medicine deserve acknowledgement for their support and patience during the period of this study. In particular, I would like to thank Miss Zoe Swann for her assistance during times of heavy sampling and Dr. Julia Kydd for the provision of help and insight when I could not see the wood for the trees. I would like to thank Dr. J.A. Mumford for the use of the department facilities and for her support throughout this project.

Throughout the course of this study both my external and internal supervisors, Dr. Ivan Morrison, Head of Immunology at the BBSRC Institute for Animal Health, Compton and Dr. Duncan Hannant, Head of Immunology at the AHT respectively, have always been there with advice and encouragement when it was needed. I would also like to thank Dr. Hannant for his continued support over the years (1986 to the present day).

Finally, my family (mainly in Newcastle) and friends (mainly in the Newmarket area) have been a constant source of encouragement and support throughout the period of my PhD studies. In particular, my wife Melanie deserves my special love and thanks for putting up with me during the writing of this thesis despite carrying our first child.

T lymphocyte responses to equid herpesviruses 1 and 4 in horses

Summary

Terry O'Neill

This thesis describes the development, optimisation and use of assays to measure equine herpesvirus-specific proliferative and cytotoxic T lymphocyte (CTL) responses in the blood of horses.

Equine T cell blast cells stimulated from peripheral blood mononuclear cells (PBMC) with pokeweed mitogen were found to perform best as targets for CTL in ^{51}Cr release assays. CTL induced *in vitro* with an abortigenic strain of EHV-1 (EHV-1/Ab4) were shown to be antigen specific, genetically restricted and predominantly of the $CD4^{-}$ $CD8^{+}$ phenotype. Cross-reactive CTL were induced *in vitro* with live EHV-4 virus, which killed EHV-1 infected blast cells. A proportion of EHV-1 induced CTL were shown to be directed against the immediate early gene products.

A proliferative LDA was used to determine whether the frequency of precursor T cells detected before challenge with EHV-1 correlated with immune status. The precursor frequency of antigen-specific T cells increased in 3 out of 4 horses after infection. However, there was no correlation between precursor frequency and outcome of infection.

A LDA was developed and used to evaluate the precursor frequencies of EHV-1 and EHV-4 induced CTL after infection with these viruses. Pre-infection CTLp frequencies in susceptible animals were <1/150,000. CTLp frequencies in animals which were immune to EHV-1 were between 1/10,000 and 1/20,000.

To my knowledge, this is the first report of the use of LDA techniques in the horse. The development and use of CTL LDA assays have provided new information on CTL responses in horses after EHV-1 and EHV-4 infection.

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1.1. The Herpesviruses

The herpesviruses are a diverse group of viruses which infect a wide range of hosts with an equally wide range of clinical and pathological consequences. Membership of the family *Herpesviridae* is based on the architecture of the virion. A typical herpesvirus is made up of an icosahedral capsid containing about 162 capsomeres, an amorphous tegument, an envelope containing a number of glycoprotein spikes and a linear double stranded DNA genome. The family is split into a number of sub-families based on the biological properties of the individual viruses (Roizmann *et. al.*, 1981). Examples of the properties used to differentiate the sub-families are; tissue tropism, genomic organisation and amino acid identity (Karlin *et. al.*, 1994) which enable researchers to classify herpesviruses into alpha (α), beta (β) and gamma (γ) sub-groups.

The *Alphaherpesvirinae* include the *Simplexvirus* (herpes simplex types 1 and 2) and *Varicellavirus* (varicella-zoster virus, pseudorabies virus, equine herpes virus types 1 and 4) genera and are classified on the basis of their short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, capacity to establish latent infections and their limited host range.

The *Betaherpesvirinae* are characterised by their long reproductive cycle and slow progress of infection in culture. This sub-family contains the *Cytomegalovirus*

(human cytomegalovirus) and the *Muromegalovirus* (murine cytomegalovirus) genera.

Members of the *Gammaherpesvirinae* replicate in lymphoblastoid cells of either the T or B cell lineage. Latent virus can be demonstrated in lymphoid cells. This sub-family contains the *Lymphocryptovirus* (Epstein-Barr virus) and the *Rhadinovirus* (herpesvirus saimiri) genera. Equine herpesviruses type 2 and 5 have been classified as gammaherpesviruses.

As this project is concerned with the immune response to the alphaherpesvirus equine herpesvirus-1, most of this thesis will concentrate on this group.

Although this system of classification is simple and easy to follow, its disadvantage is that it is not based on objective criteria. The numerous herpesvirus genomic DNA sequences which are available allow evolutionary relationships to be established between and within the herpesvirus groups. These relationships are based on conservation of genes and gene sequences between particular viruses, arrangement of the terminal sequences and the presence of methylated nucleotides (Karlin *et. al.*, 1994). This type of study has not significantly re-classified any herpesvirus to date but it has allowed the assessment of which viruses evolved before others. The study shows that the closest to the consensus genome is that of human herpesvirus-6. The closer relationships between the members of the α-herpesviruses suggests that this class is of relatively recent ancestry. In the collection of viruses studied by Karlin *et.*

al. EHV-1 was the most central α-herpesvirus suggesting that it may approximate to an ancestral α-herpesvirus.

1.1.1. The herpes simplex viruses

Primary infections of humans with herpes simplex virus type 1 (HSV-1) or herpes simplex virus type 2 (HSV-2) cause different symptoms than secondary, reactivated infections. Vesicular, ulcerative lesions of the mouth and lips are usually the consequence of primary infection with HSV-1 although the disease may be asymptomatic or include fever, sore throat, oedema, localised lymphadenopathy and malaise. Genital vesicular lesions are the most common consequence of primary HSV-2 infection. HSV-1 may also cause a less severe genital infection (Corey *et. al.*, 1983). The disease caused by primary HSV infection lasts for between 10 and 14 days after which latency is established. The establishment of the latent state depends on the existence of an intact anterior nerve route and peripheral nervous pathway (Hill *et. al.*, 1972). Latency is established when the virus retreats via the nerves innervating the site of primary infection, into the trigeminal ganglion of the infected subject. During the interim period between primary infection and reactivation, viral protein synthesis is completely shut down. It is possible, however, to grow virus from explanted ganglia co-cultivated *in vitro* on a susceptible cell line. RNA transcripts, termed latency associated transcripts, can be detected during the latent phase of HSV within human ganglia derived from autopsy material (Croen *et. al.*, 1987).

HSV shares the common herpesvirus structure mentioned above. The linear, double stranded DNA of HSV is made up of approximately 150,000 base pairs with a G+C content of 68% (HSV-1) or 69% (HSV-2). The HSV genome consists of one long unique and one short unique region linked covalently and bracketed by inverted repeat sequences. As both the long and short unique regions can invert with respect to each other, four isomers of the HSV genome exist. The genome of HSV has been fully sequenced (McGeoch *et. al.*, 1985 and 1988).

Replication of HSV proceeds with the attachment of the virus to cell receptors. Subsequently, the virus fuses with the cell membrane and the de-enveloped capsid is transported to the nucleus of the cell where the DNA is released. Transcription, replication and assembly of new capsids takes place in the nucleus. The regulation of the viral gene products after infection of a cell takes place in a coordinated manner. Viral proteins form into groups, the α, $\beta 1$, $\beta 2$, $\gamma 1$ and $\gamma 2$ proteins. These protein groups are sequentially ordered and are translated in a cascade fashion (Roizman & Sears, 1990). The synthesis of the α polypeptides, also called the immediate early (IE) proteins, reaches a peak at between 2 and 4 hours post-infection. These proteins are absolutely required for the synthesis of subsequent groups of polypeptides. The synthesis of the $\beta 1$ and $\beta 2$ proteins reaches a peak at between 5 and 7 hours post infection. The $\beta 1$ proteins include the large component of the viral ribonucleotide reductase and the major DNA binding protein. The $\beta 2$ proteins include the viral thymidine kinase and DNA polymerase proteins. The expression of these proteins signals the onset of viral DNA synthesis. The expression of the $\gamma 1$ and $\gamma 2$ proteins

depends on viral DNA synthesis. The $\gamma 1$ proteins include the glycoproteins gB and gD which are expressed relatively early in infection, whilst the $\gamma 2$ proteins, including the other glycoproteins, are expressed later in infection. There are 11 known antigenically distinct glycoproteins expressed by HSV-1. These are gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM (Ghiasi *et. al.*, 1994). Some of these glycoproteins form complexes in the envelope of the virus, for example an Fc receptor is formed via a complex between gI and gE (Johnson *et. al.*, 1988). The location of the HSV glycoproteins, on the outer surface of the viral envelope, makes them likely candidates as stimulators of the anti HSV antibody response.

1.1.2. Varicella-Zoster virus

Varicella zoster virus (VZV) is transmitted primarily by aerosolization of droplets from skin lesions. The virus initially infects the respiratory tract but then moves via the bloodstream (viraemia) to the epithelium where infection is manifest as varicella vesicles on the skin. VZV establishes latency in the satellite cells around the ganglia of the sensory neurons from the skin, on the resolution of primary infection (Hay & Ruyechan, 1994). The latent state is maintained by the host immune response. When this is compromised by, for example, age or disease VZV reactivates to cause zoster, a localised rash restricted to the skin innervated by the ganglia in which the VZV established the initial latent infection. Complications can occur during VZV infections, including varicella meningoencephalitis.

VZV DNA consists of one long and one short unique region separated by an internal repeat region. The short unique region is linked to a terminal repeat region. Isomers of this genome exist as the short unique region (and, less frequently, the long unique region) is able to invert. The VZV genome is 125,000 base pairs in size with a G+C content of 46%. The genome of VZV has been fully sequenced (Davison & Scott, 1986).

Detailed study of the replication of VZV has been hampered by the extreme species specificity of the virus. The only small animal model available, the guinea pig model, is less than ideal as only highly adapted strains of VZV can be used. From about 70 open reading frames specified in the genome of VZV, five of the encoded viral glycoproteins have been identified. These glycoproteins were termed gI, gII, gIII, gIV and gV, their HSV homologs being gE, gB, gH, gI and gC respectively. Each of these glycoproteins may play a role in stimulating anti VZV immunity (Grose, 1991).

1.1.3. Pseudorabies virus

The clinical features of disease caused by pseudorabies virus (PRV-the agent of Aujeszky's disease) depend on the age of the animal. The most acute and fatal form of the disease occurs in new born pigs and the disease is most common in 2-3 week old pigs. PRV is transmitted by droplet infection or by ingestion. The virus replicates in the nasal mucosa, tonsillar tissue and upper respiratory tract. Subsequently, it invades the lower respiratory tract and local lymph nodes from where it is disseminated via the lymph. Peripheral blood mononuclear cells (PBMC) may also

harbour the virus although cell-free viraemia is rare. In older animals the disease is characterised by encephalitis, pneumonia, resorption and mummification of the foetus and abortion. Studies have shown that mononuclear cells infected *in vitro* and re-introduced into immunised, pregnant sows caused abortion whilst cell-free virus did not (Nauwynck & Pensaert, 1992). After primary infection, the virus may become latent in the trigeminal ganglia in a similar way to HSV.

The genome of PRV has not yet been fully sequenced but it is clear that it contains a high G+C content (73%). The genome structure is similar to that of VZV. To date 8 glycoproteins have been identified in PRV. These have been correlated with those of HSV and thus consist of; gD (gp50), gE (gI), gG (gX), gI (gp63), gB (gII), gC (gIII), gH and gL.

1.1.4. The equine herpesviruses

Equine herpesviruses contain at least five recognised types, equine herpesvirus (EHV) -1, -2, -3, -4 and 5 which infect horses predominantly.

EHV-2 can be isolated from apparently healthy horses (Bagust, 1971, Harden *et. al.*, 1974). This virus is characterised by it's very slow replicative cycle, alternative designations being; slow-growing herpesvirus or equine cytomegalovirus. EHV-2 and EHV-5 were previously classified as a members of the betaherpesviridae (Roizmann *et. al.*, 1992. Browning & Studdert, 1987), although partial sequence

analysis has revealed that both of these viruses are distinct γ-herpesviruses (Telford *et. al.*, 1993).

EHV-3 causes a relatively innocuous genital infection commonly known as equine coital exanthema. The virus has been classified as an α herpesvirus and this is supported by genetic data (Baumann *et. al.*, 1986). There seems to be little serological relationship between EHV-3 and the other equine herpesviruses (Thomson *et. al.*, 1976a).

Economically, the most important equine herpesviruses are EHV-1 and EHV-4. These viruses cause a range of diseases including respiratory disease and abortion. Abortion of the equine foetus was first studied clinically by Dimock & Edwards (1932) whose further studies established a viral aetiology and defined the pathological lesions within the foetus (Dimock & Edwards, 1933 and 1936. Dimock, 1940). Manninger & Csontos, (1941) inoculated pregnant mares with bacteriologically sterile filtrates from aborted foetuses and observed influenza-like symptoms. Further studies suggested that virally induced abortion of the equine foetus was caused by infection of the mare with influenza virus (Manninger, 1949).

The respiratory disease associated with the so called equine abortion virus (EAV) was first studied in detail by Doll *et. al.* (1954b). Doll also showed that EAV was the aetiological agent causing respiratory disease in young horses and that several putative equine influenza isolates were identical to EAV (Doll *et. al.*, 1954a, Doll &

Kintner, 1954, Doll & Wallace, 1954). As the principal lesion in young horses and aborted foetuses occurred in the respiratory tract, Doll suggested that EAV be regarded as a respiratory virus (Doll *et. al.*, 1957). In this study, the authors designated the disease caused by EAV as equine rhinopneumonitis and the virus as equine rhinopneumonitis virus. EAV was later designated as EHV-1 subtype 1 (the abortogenic strain) and subtype 2 (the respiratory strain, Matthews, 1979).

EHV-1 (old designation) was recognised as causing four distinct syndromes; respiratory disease, abortion, neonatal foal disease and occasionally, paralysis (Mumford, 1994). In an extensive study of the capacity of the two subtypes of EHV-1 to cause disease, Allen & Bryans (1986) showed that the vast majority (all but one) of the abortion epizootics studied were caused by subtype-1 viruses and only this virus was ever isolated from cases of central nervous system disease. However, both subtype-1 and subtype-2 viruses were isolated from outbreaks of respiratory disease, although the majority were subtype-2 (86%). The distinct restriction endonuclease analysis patterns of the two sub-types suggested that they should be regarded as separate viruses (Studdert *et. al.*, 1981). The latest International Committee an the Taxonomy of viruses (ICTV) suggests the use of EHV-1 for the former subtype-1 virus and EHV-4 for the former subtype-2 virus, based on the differences outlined above (Roizman *et. al.*, 1992).

Comparative studies between EHV-1 and EHV-4, using immunofluorescent techniques, showed that EHV-4 replicated in the epithelium of the nasopharynx and

the bronchial tree, macrophages and lymph nodes only. In contrast, EHV-1 replicated in these sites as well as the endothelial lining of the vasculature of the nasopharynx and the lung (Patel *et. al.*, 1982, Patel & Edington, 1983). EHV-1 was also found in circulating leukocytes whereas EHV-4 was not. The pathogenesis of EHV-1 and EHV-4 will be discussed in more detail later in this chapter.

As with all other herpesviruses, EHV-1 and EHV-4 become latent after the primary infection. Unlike HSV and PRV, there is evidence that EHV does not become latent in sensory ganglia (Allen & Bryans, 1986, Edington *et. al.*, 1985). However, recent studies dispute this (Slater, 1994) and suggest that EHV-1 may, indeed, be neurotropic. The site or sites of latency of EHV have not yet been conclusively identified.

The genome of EHV-1 has been fully sequenced (Telford *et. al.*, 1992) has been shown to consist of approximately 150,000 base pairs, with a G+C content of 56 or 57% (Whalley *et. al.*, 1981, Soehner *et. al.*, 1965). The DNA is divided into one long and one short unique region flanked by inverted and terminal repeat regions in a similar arrangement to the genomes of PRV and VZV (Ben-Porat *et. al.*, 1979, Davison *et. al.*, 1984).

The structure of the EHV virion is typical of the other members of the herpesviridae. It has an icosahedral capsid enclosing the DNA core. This is surrounded by a loose envelope which is derived from the nuclear membrane of the infected cell. The

envelope is a triple layered membranous structure which contains lipid, phosphoproteins and glycoproteins. The viral glycoproteins of EHV-1 and EHV-4 are the most immunodominant antigens, at least in terms of antibody production (Tewari *et. al.*, 1993). There are six major glycoproteins (in terms of abundance, Allen & Yeargan, 1987) specified by EHV-1 and EHV-4. Some of these have equivalents in HSV whilst others do not. The EHV glycoproteins are referred to as follows (the HSV equivalents are in parenthesis); gp2 (-), gp10 (gH), gp13 (gC), gp14 (gB), gp18 (gD) and gp21/22a (-).

1.2. Immune responses to herpesviruses

1.2.1. Introduction

An animal's defence against foreign material entering the body and causing pathological change consists of a wide range of innate and adaptive mechanisms.

One of the most effective defense mechanisms is the physical barrier formed by the skin and mucosal surfaces. The mucocilliary escalator in the trachea and the acid environment of the stomach also form barriers against entry of infectious material. If these primary barriers are breached and the foreign material enters the internal environment of the animal then the immune system is activated in an attempt to eliminate the potential threat.

An infectious agent which has entered the body becomes a focus for inflammatory and innate immune responses, which are triggered immediately when a microbial challenge is encountered. These responses may involve tissue macrophages, monocytes, neutrophils, natural killer cells (NK cells) and endothelial cells and also cytokines, complement and arachidonic acid metabolites. Tissue macrophages, which develop from blood monocytes, are able to phagocytose pathogens via interactions with receptors such as the mannose-fucose and complement receptors. Actively phagocytic tissue macrophages become stimulated to produce a number of products which are capable of inducing the expression of adhesion molecules which enhance the interaction of neutrophils and endothelial cells. This allows the egress of neutrophils from the blood into the tissues down a concentration gradient of numerous chemotactic factors. These peptide factors, produced by macrophages and endothelial cells, also have strong neutrophil arming properties which enables the neutrophils to phagocytose and kill bacteria using a number of highly toxic proteinases and reactive oxygen intermediates. On completion of their main function, the neutrophils die and are rapidly removed from the tissues via phagocytosis by macrophages.

NK cells are able to kill certain tumour or virally infected target cells *in vitro*, in a non-genetically restricted manner. They often appear as large granular lymphocytes (LGL) and they do not produce a secondary, memory response. However, they are able to lyse cells quickly after challenge with some infectious agents and have been implicated as a natural resistance mechanism in some herpesviral systems.

As an infectious episode progresses the components of the adaptive immune system begin to have an effect. Specific antibody is produced against the pathogen by B cells which differentiate, under the control of helper T cells, into antibody producing plasma cells. Effector Cytotoxic T lymphocytes (CTL) differentiate from precursors and lyse virally infected cells in a genetically restricted, antigen-specific manner. Cytokines are produced by T cells, which are able to modulate the immune response to the pathogen and also inactivate certain viruses directly.

The outcome of a herpesvirus infection depends on the immune status of the host when the virus enters the body. If no pre-existing immunity exists then it is likely that the virus will cause pathological change and illness until components of the adaptive immune system can be activated and eliminate the virus or force it to become latent. If however, the animal has had a recent exposure to the same virus, then not only will pre-existing immunity increase the chances of inactivating the virus before it establishes an infection but the increased speed and magnitude of the secondary response will have an earlier effect on established infection by preventing its spread. As herpesviruses have co-evolved with the immune systems of animals, numerous escape mechanisms which ensure their survival have developed. Probably the most effective of these escape mechanisms is their ability to become latent. This is a common property of all herpesviruses and enables the virus to persist in the host until favourable conditions exist for its re-activation.

What follows is an overview of the mechanisms involved in the attempted elimination of viruses and viral antigens from animals infected with herpesviruses. As this study is concerned with equine herpesvirus type 1 (EHV-1), an α-herpesvirus, the majority of the work described in the text will concentrate on this class of virus.

1.2.2. Innate immune responses

The innate immune system exists to provide a first line of defence against infectious challenge. As outlined above, it consists of specialised cells capable of mounting a rapid response to a challenge without having previously encountered the antigens of the pathogen. The majority of the work on natural resistance to herpesvirus infections has been carried out in mouse models. Inbred mouse strains have been identified which are either resistant, moderately susceptible or susceptible to HSV infection (Lopez, 1975).

1.2.2.1. Macrophages

Phagocytosis is an important process in the initiation of an innate response. The phagocytic cells present in the tissues are members of the mononuclear phagocyte or macrophage family. Macrophages are present in all major tissues of the body. Tissue macrophages are derived from blood monocytes which migrate into the tissues and become one of a number of specialised tissue macrophage type cells e.g. microglial cells of the brain, Kupffer cells in the liver and pulmonary macrophages in the lung.

After gaining entry into the tissues a herpesvirus will immediately come into contact with phagocytic tissue macrophages. Depending on a number of factors, an infectious state will be established or, if the animal is naturally resistant to the virus, there will be no infection. If the macrophage phagocytoses the virus, then the internal environment of the lysosome may be enough to inactivate it. This is the case when C57BL (HSV resistant) mice are infected with HSV-1 (Sarmiento *et. al.*, 1988). Bone marrow, peritoneal, and splenic macrophages from these mice consistently inhibit HSV-1 replication before the onset of immediate early gene expression or viral protein synthesis. Molecular analysis of "infected" resistant peritoneal macrophages from resistant mice (Morahan *et. al.*, 1989) indicated that the immediate early and early thymidine kinase (tk) genes were expressed whilst transcripts for the early DNA polymerase, delayed early glycoprotein D and the late glycoprotein C genes were not detectable. This suggests that there is a block in virus replication in macrophages from resistant mice which lies between the expression of the early to delayed early viral genes.

The activational state of the macrophage also influences the outcome of infection with HSV-1. Tenney *et. al.* (1987) showed that undifferentiated macrophage like cells were non-permissive to HSV-1 infection whereas the same cells activated with phorbol esters (PMA) became permissive to infection with the same virus. Mouse peritoneal macrophages stimulated with *Corynebacterium parvum* or thyoglycolate showed increased levels of productive infection, cytopathic effect and DNA replication when compared with resident macrophages (Sit *et. al.*, 1988).

The resistance of macrophages to HSV infection may also be due to their high secretory potential. Resident macrophages can be stimulated to secrete a wide range of molecules, for example, interleukin 1 (IL1), interleukin 6 (IL6), eicosanoids, interferon alpha (IFNα) and tumour necrosis factor alpha (TNFα). The infecting virus may also act as a physiological activator of macrophages (Giridhar *et. al.*, 1991). Proteolytic enzymes and reactive oxygen intermediates produced by macrophages can directly inactivate extracellular particles and IFN may inhibit viral replication in surrounding cells. The importance of IFN in protection of cells against HSV infection was indicated by Pyo *et. al.* (1991) who showed that resistance of inflammatory macrophages to infection with HSV-1 induced by the IFN inducer polyriboinosinic:polyribocytidylic acid (poly I-C) was completely abrogated by antibody to interferon beta (IFNβ). Interferon gamma (IFNγ) present in supernatants from T helper cell clones was implicated in increasing the *in vitro* anti-HSV virucidal activity of macrophages (Seid *et. al.*, 1986). Current nomenclature refers to IFNα and IFNβ (produced by a wide range of cell types) as type I IFNs and IFNγ (produced by activated T cells and NK cells) as type II. Recently, Muller *et. al.* (1994) have published results of studies on the role of the IFN system in antiviral defence. Mutant mice with deleted IFN receptor genes (either type I or type II IFN receptors) were infected with vesicular stomatitis virus (VSV), Semliki Forest virus (SFV), vaccinia virus and lymphocytic choriomeningitis virus (LCMV). Comparison of mice lacking either type I or type II IFN receptors showed that, at least in some of these infections, both IFN systems were essential for antiviral defense and they were functionally non-redundant.

The only report of experiments to test whether mononuclear phagocytes are important in protection against EHV-1 infection was published by Darlington (1978) who demonstrated that macrophages from yearling horses supported the replication of EHV-1 whilst those from older animals did not. Macrophages from the older horses were resistant to infection with EHV-1, however, abortogenic infections and viraemias did occur suggesting that macrophages did not protect against these very important consequences of infection.

1.2.2.2. Natural Killer (NK) cells

NK cells were first described as an unwanted background in studies of antigen specific T cell mediated cytolysis of tumour target cells. Since then they have been widely studied as a component of the natural immune response against viral infections. They are characterised by their ability to lyse various tumour cell lines (eg. K562 cells) and virally infected (eg. HSV infected fibroblasts) targets *in vitro* without previous experience of the expressed tumour or viral antigens and in a non-MHC restricted manner. NK cell function can be augmented *in vitro* by IL2 (Shaw *et. al.*, 1985) and IFN (Colmenares and Lopez, 1986).

NK cells have largely been defined in terms of their function (reviewed by Reynolds & Ortaldo, 1987). In both mice (Lust *et. al.*, 1981) and humans (Fitzgerald *et. al.*, 1983) NK cell functional activity has been shown to be due to a heterogeneous population of cells. This heterogeneity is manifest by the differential lysis of NK sensitive target cells by NK cells. For example, NK cells from one patient may lyse

HSV infected fibroblasts but not K562 cells whereas NK cells from another patient may do the opposite, suggesting that there are at least two populations of NK cell (Fitzgerald *et. al.*, 1983).

All mouse NK cells can be characterised as low density lymphocytes (Bukowski & Welsh, 1985) and are within the large granular lymphocyte (LGL) population (Minato *et. al.*, 1985). Depending on the species, NK cell identification varies, for example, porcine NK cells are low density lymphocytes but not LGL (Yang *et. al.*, 1987) whereas bovine NK cells lack the large intracytoplasmic granules identifying human LGL (reviewed in Cook & Splitter, 1989). Phenotypically, human NK cells are characterised by the expression of CD16 (Fc receptor γ IIIa/b) and CD57 (NKH-1) and the lack of expression of CD3 and the T cell receptor. A sub-set of human NK cells also expresses CD8 and CD2 although the functions of these molecules, which are found mainly on T cells, on NK cells is uncertain although they may suggest a common lineage between these cell types. Murine NK cells are defined by the expression of NK1.1 and asialo GM1 on the cell surface.

Most viral infections, including HSV-1 and HSV-2, augment NK cell function 1-3 days after infection. Welsh *et. al.* (1978) showed that this augmentation followed closely the generation of IFN in the mouse. However, Fitzgerald *et. al.* (1982) showed that NK cell lysis of HSV infected fibroblasts did not correlate with the amount of IFN-α produced in the assay. They also showed that, in some humans, NK cell activity against HSV infected fibroblasts was normal despite the generation of

only small amounts of IFN. In a mouse model of acute ocular HSV-1 infection, Brandt and Salkowski (1992) showed that, using a natural route of infection, splenic NK activity was augmented after infection. They also assessed the role of IFN and IL2 in the NK cell activation by treating the mice with antibodies to IFN-α/β, IFN-γ and IL2 before infection. Treatment with anti IFN-α/β or IFN-γ resulted in reduced NK cell cytotoxicity whereas treatment with anti IL2 resulted in increased cytotoxicity suggesting that *in vivo* IFN is important in NK cell activation whilst IL2 is involved in suppression of NK cell activation.

The role of NK cells in limiting the replication of HSV-1 was studied by Leibson *et. al.* (1986) who found that cytotoxic NK cells reduced virus yield from infected fibroblasts by up to 90%. There was an inverse relationship between the number of NK cells in the culture and the virus yield. Experiments using murine cytomegalovirus (MCMV) infection have demonstrated the important role of NK cells in recovery from this disease. Beige mice were found to have low NK responses and were more susceptible to severe herpesvirus infections than normal litter-mates. Replication of MCMV was also enhanced in young mice and mice depleted of NK cells using anti-NK1.1 and anti-asialo GM1 antibodies (Welsh & Vargas-Cortes, 1992). An antiviral role for NK cells has also been demonstrated in mice with severe combined immunodeficiency (SCID), indicating that they are able to function in the complete absence of T or B cells (Welsh *et. al.*, 1991). The role of NK cells in resistance to herpesvirus infections has also been studied in humans. Biron *et. al.* (1989) described a patient with a complete NK cell immunodeficiency who

succumbed to exceptionally severe CMV, VZV and HSV-1 infections. Other studies have also linked low NK cell activity to severe cases of HCMV (Quinnan *et. al.*, 1982) and HSV (Lopez *et. al.*, 1983).

NK cells have not been extensively studied in the horse. The only report of equine NK activity was published by Chong *et. al.* (1992) who demonstrated that NK cell activity was not enhanced after EHV-1 infection of specific pathogen free (SPF) foals.

1.2.2.3. Antibody dependent cell mediated cytotoxicity

Antibody-dependent cell-mediated cytotoxicity (ADCC) is facilitated when antibody binds specifically to antigen expressed on the surface of a target cell and non-specifically to Fc receptors on the surface of an effector cell. Any effector cell capable of participating in the ADCC reaction must, therefore, express at least one type of Fc receptor on its surface. The cells involved in mediating ADCC are usually termed killer (K) cells although they consist of a heterogeneous group of leukocyte effector cells including LGLs, a small proportion of T cells and some macrophages. The major isotype of antibody responsible for ADCC function, at least in the HSV system, is IgG. Methiesen (1988) found that anti HSV-1 ADCC mediating activity was detectable mainly in the IgG1 and IgG3 fractions. In order for effective ADCC to occur, molecules other than immunoglobulin need to participate. For example, Kohl *et. al.* (1986) showed that patients genetically deficient in the leukocyte cell surface glycoproteins CD11a (LFA-1), CD11b (Mac-1) and p150,95 were unable to provide leukocyte effector cells able to mediate ADCC *in vitro*. The main emphasis of the

published literature concerning ADCC has been in neonatal HSV infections. Both human (Kohl *et. al.*, 1984) and murine (Kohl & Loo, 1980) neonates are potentially deficient in anti HSV ADCC activity. A correlation between level of ADCC antibody and HSV disease has been demonstrated in human neonates (Kohl *et. al.*, 1989). In particular, individuals with disseminated disease were shown to have significantly lower levels of anti-HSV ADCC mediating antibody than those with localised disease. The implication from these studies is that ADCC is important in controlling neonatal HSV infections and that passively acquired maternal antibody participates in the ADCC reaction.

1.2.3. The role of antibody in herpesvirus immunity

The role of anti herpesvirus antibodies in protection against, and recovery from infection has been partially covered with the discussion of the ADCC reaction in the previous section (see 1.2.2.3.). Many studies have concluded that the general mechanism of antibody mediated protection against herpesvirus infection is via ADCC. For example, Hayashida *et. al.* (1982) concluded that antibody mediated protection from HSV infection of athymic nude mice required the Fc portion of the antibody molecule implying that ADCC was involved. Balachandran *et. al.* (1982) concluded that the protection of complement-deficient and normal mice with non-neutralising monoclonal antibodies specific for HSV-1 gC, gB, gD, gE and gF was due to ADCC. Kapoor *et. al.* (1982b) showed that passive transfer of polyclonal neutralising antibody to nude mice resulted in a reduction in the level of virus in ganglia. When monoclonal antibodies were transferred to these mice only one

neutralising antibody reduced virus spread to the ganglia which, the authors suggest, argues in favour of neutralisation *per se* being the mechanism in use. All of these studies were based on the passive immunisation of animals with polyclonal or monoclonal antibody preparations which may not reflect the levels of antibody present in normal mice after an ongoing immune response. In a more natural situation Mishkin *et. al.* (1992) immunised normal mice with HSV-1 gD, depleted of various cell types and then challenged with HSV-2. After challenge of these animals with low to moderate levels of HSV-2, vaccine elicited antibody played a primary role in limiting the severity of infection whereas at high challenge levels T cell mediated mechanisms became important. Analysis of a large number of herpes simplex virus type 1 or 2 infected patients by Reeves *et. al.* (1981) revealed that among patients with primary HSV-2 infections, the probability of recurrence directly related to the presence and level of anti HSV-2 neutralising antibody. This result, based solely on antibody measurements, does not preclude the presence of other protective mechanisms within the same patients.

The majority of studies concerned with the role of antibody in protection against herpesvirus disease have used circulating antibody as a measure of protective potential. However, there is a growing body of evidence implicating mucosal antibody as a major determinant of anti-herpesvirus immunity. Irie *et. al.* (1993) showed that mice could be protected against ocular and cutaneous infection with herpes simplex virus type 1 by intra-gastric immunisation with live virus. A mucosal barrier against infection with bovine herpesvirus 1 (BHV-1) was achieved when calves were

immunised with BHV-1 viral envelope glycoproteins combined with the potent mucosal adjuvant cholera toxin beta subunit (Israel *et. al.*, 1992).

1.2.4. Specific cellular responses

Although cellular mechanisms are involved in all aspects of innate and specific immunity to infectious agents, the term cell-mediated immunity (CMI) has come to be synonymous with the T cell immune response.

Mature T cells are bone marrow derived cells which express the clonotypic, T cell receptor (TcR) complex which includes the CD3 signal transduction apparatus. Immature T cell precursors migrate from the bone marrow to the thymus where, as pro-T cells, they undergo a process of division and differentiation during which they are educated to recognise antigenic peptides embedded in the peptide binding groove of either MHC class I or MHC class II proteins. Both MHC class I and MHC class II proteins are specialised polymorphic peptide binding molecules. Class I proteins consist of a 45kD membrane bound heavy chain non-covalently linked to a 12kD polypeptide termed β2-microglobulin (β2M). The class I heavy chain can be divided into three extracellular domains, the α3 domain is membrane proximal and has homology to an immunoglobulin domain whilst the α1 and α2 domains contain extensive intraspecies polymorphism and form a peptide binding groove. Class II proteins are non-covalently linked αβ heterodimers (α chain 33kD, β chain 29kD). Both chains are membrane bound and the peptide binding groove is formed between

the $\alpha 1$ and the $\beta 1$ domains. The majority of the polymorphism in MHC class II molecules is in the $\beta 1$ domain.

1.2.4.1. Thymic education

On entering the thymus from the foetal liver, spleen or bone marrow, the developmentally immature pro-T cells undergo a process by which they are either eliminated if their TcR has high affinity for self MHC (negative selection, Kappler et. al., 1988) or they are selected if the TcR has an intermediate affinity for self MHC (positive selection, Blackman et. al., 1989). This ensures that, whilst T cell responses against self proteins are prevented, TcR molecules retain the capacity to recognise self MHC and distinguish it from self MHC containing foreign peptide. The cell which leaves the thymus expresses high levels of the TCR/CD3 complex and either CD4 or CD8. The CD4 and CD8 molecules have similar functions in that they both act as accessory molecules by stabilising the interaction between MHC molecules and the TcR. These molecules also act as co-receptors by transducing a signal via the tyrosine kinase, $p56^{lck}$ which associates with the cytoplasmic domains of both molecules (Veillette et. al., 1989). The cells which leave the thymus are referred to as the naive T cell pool. An extra-thymic maturation process for some T cells has also been suggested. Recent evidence has implicated gut-associated lymphoid tissue in the extra-thymic maturation of intra-epithelial lymphocytes of the small intestine (Lefrancois & Puddington, 1995). After the thymic education process T cells are only able to recognise antigens in the form of MHC protein-peptide. The obligate recognition of MHC bound peptide antigen is termed MHC restriction.

1.2.4.2. Antigen processing

Naive T cells continually re-circulate between the peripheral lymphoid tissues via lymphatics and the blood. This ensures that an individual T cell comes into contact with a large number of antigen presenting cells (APC) which present peptide fragments within their cell surface MHC molecules. These peptide fragments are derived from the internal environment of the APC (self or viral peptides) and from any foreign material which enters the body and drains into the lymphoid tissue where it can be taken up by APCs.

The mechanisms by which APCs derive antigenic peptide from native antigens and present them to T cells has been intensively studied in recent years. Studies using the mutant cell line RMA-S (Townsend, 1989), which has a defect in the association of β2M with the class I heavy chain, have shown that the folding of Class I molecules is dependent on the availability of peptide. This means that Class I-peptide interaction must take place soon after the biosynthesis of the Class I molecule. The endoplasmic reticulum (ER) has been shown to be the site of class I-peptide interaction (Yewdell & Bennink, 1989). The major source of peptide for this interaction is the cytoplasm. Therefore, it is clear that peptides must enter the ER in order to associate with class I molecules. Recent studies have identified two genes in the MHC region (Trowsdale et. al., 1990) that code for the heterodimeric ER membrane bound protein components TAP-1 and TAP-2 which have a peptide transporting function (Powis et. al.,1992). The mechanism of generation of the peptides which become available for transport into the ER is not clear. However, a number of MHC linked genes which encode low

molecular weight proteins, the LMP genes, and which are closely linked to the TAP genes encode proteins with homologies to molecules contained in multicatalytic proteases assembled in structures termed proteasomes (Goldberg & Rock., 1992). Two LMP genes (LMP2 and LMP7) have been shown to modify the peptidase activity of proteosomes towards the production of peptide able to bind to MHC class I molecules (Gaczynska *et. al.*, 1994). Thus, it is possible that proteins derived from intracellular events are produced in the cytoplasm of APCs where they may be degraded by multicatalytic proteasomes into peptides of optimal length for transport across the ER membrane by TAP-1 and TAP-2 after which they combine with the newly synthesised MHC class I proteins.

Processing of antigen for presentation in the context of MHC class II molecules begins with endocytosis of extracellular antigen. The processing of exogenous antigen is rapid and efficient and is generally inhibited by agents which interfere with the acidification of endosomes. It has also been shown that class II molecules bind peptides later after their biosynthesis than do MHC class I molecules (Germaine, 1986). This delay in peptide binding is a function of the more stable, two membrane bound chain, structure of class II molecules and the fact that they are "protected" from peptide binding in the ER by their association with a molecule known as the invariant chain (Ii). Ii also facilitates dimerisation of class II and regulates the export of intact molecules to the cell surface. Class II-Ii complexes move from the Golgi through early endosomes after which they accumulate in late endosomes/pre-lysosomes. Ii is removed in this compartment due to the acidic and proteolytic environment which

makes the class II available for the binding of peptides which are also produced at this site. The removal of the Ii in the late endosomes/pre-lysosomes allows the class II-peptide complexes to escape from the endosomal pathway and become expressed on the cell surface.

1.2.4.3. T cell activation

The sampling of peptide specificities via interaction of the TcR with MHC-peptide on the surface of APCs is facilitated by a range of adhesion molecules on the surface of the T cell which have ligands on the APC surface. Thus, naive T cells work their way through lymphoid tissues always in full contact with other cells via the interactions between LFA-1 (binds ICAM-1, -2 and -3) and CD2 (binds LFA-3) on the T cell surface and between ICAM-1 (CD54), ICAM-2 and ICAM-3 (CD50) and LFA-3 (CD58) on the APC surface.

The majority of these interactions do not result in T cell activation. TcR recognition of a specific peptide is not sufficient for T cell activation and this event in isolation results in T cell tolerance. In order for T cell activation to occur TcR ligation must be accompanied by the delivery of a co-stimulatory signal. This level of regulation ensures that only interactions between antigenic peptide presented on the surface of professional APCs trigger naive T cells to become activated and develop into effector T cells. When an antigenic peptide is recognised by a re-circulating T cell and the co-stimulatory signal is delivered, adhesion to the APC presenting the peptide is enhanced and T cell activation ensues.

The APC type on which the antigenic peptide was presented to the T cell is crucial in determining the outcome of the interaction. For example, dendritic cells are thought to be the only APCs able to activate naive T cells and thus trigger true primary responses. Human dendritic cells express both MHC class I and MHC class II molecules, ICAM -1, -2, and -3, LFA-3 and the co-stimulatory molecule B7. Other professional APCs, for example macrophages and B cells do not constitutively express co-stimulatory (B7) activity and need to be activated before being able to function fully as APCs.

Activation of a T cell begins with the binding of the TcR to MHC/peptide and the binding of the co-stimulatory signal B7 (and the related molecule B7.2) to its ligand CD28 (also CTLA-4 on activated cells which binds B7 with a 20 fold higher avidity than CD28). After these events occur, proliferation of the T cell ensues which is driven by the production of the autocrine T cell growth factor interleukin 2 (IL2) and the expression of its receptor CD25. The co-stimulatory signal has the function of stabilising the production of IL2 and inducing proliferation of the T cell which dilutes the inhibitory signals that accumulate when the TCR is ligated in isolation (Jenkins, 1992).

1.2.4.4. T cell effector functions

After a period of proliferation the T cells differentiate into one of a number of effector T cells. Effector T cells differ from naive T cells in that they can respond to antigenic peptide presented in the absence of any co-stimulatory signal which means that they

can have an effect in tissues which do not express B7. They also express higher levels of the adhesion molecules CD2 and LFA-1 and begin to express the vascular endothelial homing receptor VLA-4 and the CD45RO isoform of CD45. This means that effector T cells become more sensitive to further stimulation with MHC bound specific antigenic peptide.

There are two major types of effector T cell, the helper T cell and the cytotoxic T cell. These cells are often differentiated from each other by their expression of CD4 and CD8 respectively. The terms helper and cytotoxic T cells are, however, functional descriptions and, at best, their designation as $CD4^+$ and $CD8^+$ respectively is misleading. The CD4 and CD8 molecules expressed on the cell surface of T cell sub-sets correlate well with the MHC reactivity of the particular T cell; thus, CD4 binds specifically to MHC class II molecules whilst CD8 binds to MHC class I molecules.

T helper (Th) cells are mainly involved in the regulation of immune responses via the production of soluble cytokines. An *in vivo* consequence of the production of cytokines by Th cells is a reaction known as delayed type hypersensitivity (DTH) which has been used as an indication that cellular immune mechanisms are at work in model systems. Th cells can be divided into two distinct sub-populations based on their cytokine secretion profiles (Mossmann et. al., 1986). Type 1 helper cells (Th1) produce IL3, GM-CSF, IL2, IFN γ and lymphotoxin. Type 2 helper cells (Th2) produce IL3, GM-CSF, IL4, IL5, IL6 and IL10.

Th1 cells produce cytokines which are involved in the mediation of DTH reactions and in the activation of macrophages which are then able to kill intracellular or extracellular pathogens (Cher & Mossmann, 1987). Some of the cytokines produced by these cells (e.g. TNF and IFN-γ) also have direct cytotoxic effects on cells. Th2 cells have the main function of helping B cells to produce high levels of specific antibody. Some IgM responses and all other Ig responses are T (predominantly Th2) cell dependent. Thus, in the mouse, IL4 induces a switch in antibody production by B cells from IgM to IgG1 and IgE and TGF-β induces a switch to IgG2b and IgA. Help with antibody production is not the exclusive preserve of Th2 cells as some Th1 cytokines induce an isotype switch, e.g. IFN-γ induces a switch from IgM to IgG3 and IgG2a synthesis. A third type of T helper cell has been described, the Th0 cell. These cells produce a mixture of Th1 and Th2 cytokines and are said to be the precursors of these cells.

Most of the research carried out on T helper cell sub-sets has used T cell lines or clones from mice. However, there is growing evidence for the existence of similar sub-sets in rats and in humans.(Noble *et. al.*, 1993, van-der-Pouw-Kraan *et. al.*, 1993)

In addition to their positive effects on the immune response, Th cells have negative or inhibitory effects. Thus, Th1 cells inhibit the growth and differentiation of B cells stimulated by IL4, a Th2 cytokine. Conversely, IL4 and IL10, both Th2 cytokines, inhibit IFN-γ induced macrophage killing of pathogens. Th cell sub-sets also have inhibitory effects on each other. IFN-γ has been shown to inhibit the growth of

murine Th2 cells whilst IL10 and, to some extent IL4, can reduce cytokine production by Th1 cells (Fiorentino et. al., 1989).

The in-vivo significance of T helper cell sub-sets to the resolution of disease processes can be illustrated by studies of *Leishmania major* infection of mice. Infection of most strains of in-bred mice with this parasite results in the production of localised lesions which resolve within two weeks, resulting in a state of resistance to re-infection. The response of these mice is characterised by a strong DTH response but only low levels of antibody. Cells derived from these mice produce high levels of IFN-γ but little or no IL4 or IL5. This is therefore a typical Th1 type response (Belosevic et. al. 1989). However, when BALB/c mice are infected sub-cutaneously with the same parasite the resultant localised lesions spread, resulting in disseminated disease and death. High levels of antibody are produced along with a low DTH response. Cells derived from these mice produce high levels of IL4 and IL5 but little IFN-γ. This is a typical Th2 response. It is known that the most effective mechanism for the killing of *L.major* parasites is via activated macrophages, which are primarily induced by Th1 cells. This example illustrates the consequence of an inappropriate immune response to a pathogen.

The activation of naive $CD4^+$ and $CD8^+$ T cells is essentially the same except that $CD4^+$ T cells are stimulated by antigen presented by MHC class II molecules whilst $CD8^+$ T cells are stimulated by antigen presented by MHC class I molecules. Cytotoxic T lymphocytes (CTL), sometimes referred to as $CD8^+$ T cells, differ from

helper T cells, sometimes referred to as $CD4^+$ T cells, in their effector functions within target tissues.

The primary effector function of CTL is the lysis of cells infected with intracellular pathogens, e.g. all viruses, some bacteria and a few parasites. The display of endogenously processed peptide antigens within the cell surface MHC class I molecules of the infected cells makes the cell "visible" to antigen specific effector CTL and hence susceptible to lysis. This situation is analogous to the presentation of antigen to naive CTL by, for example, dendritic cells, which results in its differentiation into an armed effector CTL.

1.2.4.5. Cytotoxic mechanisms

The end result of an encounter between an infected cell and an effector CTL is usually the destruction of the infected target cell. The initial recognition event between the target cell and the effector CTL involves linkages between antigen specific TcR and the MHC-peptide molecule. This is followed by the numerous adhesion molecule interactions described above, which are critical for efficient target cell killing. After the recognition event, target cell killing can be mediated using one of a number of proposed mechanisms which are probably not mutually exclusive.

The first mechanism is receptor mediated triggering of apoptosis. Apoptosis is a normal cellular mechanism which, for example, deletes all thymocytes that fail positive selection or have a high affinity to self MHC in the thymus (Smith *et. al.*,

1989). The process of apoptotic cell death involves DNA fragmentation, cytoplasmic streaming, membrane blebbing and the eventual destruction of the cell. The initiation of apoptosis in target cells via cross linking of cell surface receptors by effector CTL is accompanied by an increase in intracellular Ca^{2+} (McConkey *et. al.,* 1989). It has been suggested that the rise in intracellular Ca^{2+} initiates the action of endogenous endonucleases which bring about pre-lytic DNA fragmentation, although the link has not been shown experimentally. The Fas/Fas ligand interaction plays a role in the initiation of apoptotic cell death in a number of systems (Nagata & Suda, 1995). Fas ligand is a factor which binds its receptor, Fas (CD95), and induces cell death by apoptosis. A role for this interaction in CTL killing has been suggested as CTL hybridomas have been isolated which kill Fas^{+} cells but not Fas^{-} cells (Rouvier *et. al.*, 1993). Whatever the mechanism, DNA fragmentation occurs followed by membrane blebbing and cell lysis. CTL DNA fragmentation can be detected within 10-15 minutes after effector-target cell binding. Any intracellular viral DNA present during this process may also be degraded by the endogenous endonucleases (Martz & Howell, 1989). Although this model was first described by Russell in 1983 the triggering mechanism and the target cell "second messenger" responsible for the induction of DNA fragmentation has still to be defined.

The second model of CTL mediated lysis of target cells is the granule exocytosis model. The initial steps of this model are the adhesion between granular CTL and the target cell, and the polarization of the CTL cytoplasm towards the target cell. This polarization allows the CTL to deliver a lethal hit to the target cell without affecting

the surrounding cells. The lethal hit consists of the secretion of the pre-formed contents of cytoplasmic granules into the gap junctions between the CTL and the target cell. Isolated granules from CTL and NK cells contain a 65kD protein called perforin (or cytolysin), hydrolytic lysosomal enzymes, several serine proteases (granzymes) and proteoglycans. The perforin molecule is able to polymerise in the presence of Ca^{2+} to form hollow tubular structures which span the membrane of the target cell. Perforin is antigenically related to the ninth component of complement (C9). After the polymerization of perforin and the subsequent pore formation, serine protease influx into the target cell has been implicated in the observed DNA breakdown. The evidence for this comes from experiments in which DNA lytic activity was inhibited using protease inhibitors and target cells were lysed using mixed, purified preparations of serine protease and perforin (Hayes *et. al.*, 1989). Recently, the granule exocytosis model of CTL killing has been shown to be important *in vivo* by the development of perforin deficient mice (Kagi *et. al.*, 1994). These mice have normal numbers of both $CD8^+$ cytolytic T cells and NK cells which do not lyse virus infected or allogeneic fibroblasts or natural killer target cells (*e.g.* YAC-1 cells) *in vitro*. However, cytolytic activity against LCMV peptide labelled RMA lymphoma cells was observed using cells from these mice, which suggests that a second cytolytic mechanism exists. This residual cytotoxic activity appeared to be mediated by Fas ligand (Lowin *et. al.*, 1994)

1.2.4.6. Immunity to herpes simplex virus in the mouse

Innate mechanisms represent a formidable barrier to infection with herpesviruses (see section 1.3.1.). However, if this barrier is breached and infection becomes established, adaptive immune responses, in particular T cell responses, play a major role in elimination of virus from infected tissues and subsequent recovery from infection. A large body of work has been produced in which basic mechanisms of T cell immunity to most of the herpesviruses have been studied.

The most widely studied virus in the herpesvirus group is the prototype α-herpesvirus, Herpes Simplex virus (HSV). One of the reasons for the predominance of HSV research is the existence of well characterised animal models of human disease. The HSV mouse model is, in fact, a collection of different models some of which resemble HSV infection in humans. The main differences between murine and human HSV infection are that systemic infections of mice usually result in death of the animal and secondary and recurrent infections cannot be demonstrated in the mouse. It is clear that, if a disease model is to be used experimentally, then attempts must be made to ensure that it has some relevance to the host animal. For example, experiments using the intra-peritoneal (IP) route of infection show little resemblance to the muco-cutaneous route seen in humans, are very much influenced by innate immune mechanisms and give little quantitative information on the growth of HSV as the end point is death; therefore the usefulness of the IP mouse model is questionable. However, the ear clearance (EC) or the footpad clearance (FC) mouse models allow quantitative assessments of the level of virus growth in the tissues of the animal and

the rate of clearance of the virus after adoptive transfer of virus specific antibodies or immune cells after challenge. Despite these problems, the HSV mouse models have proven to be reliable tools for the delineation of basic mechanisms involved in immunity to HSV.

Initial, adoptive transfer experiments showed that HSV immune T cells protected X-irradiated and anti-thymocyte antibody treated mice against an otherwise lethal HSV infection (Oakes, 1975). This was confirmed when adoptive transfer of immune T cells to congenitally athymic (nude) mice protected them against lethal HSV infection whereas transfer of neutralising antibody merely prolonged the survival time of the mice (Nagafuchi *et. al.*, 1979). Later it was shown that neutralising antibody does play a role in anti HSV immunity in mice when Kapoor *et. al.* (1982a) showed that HSV infection of B cell suppressed mice resulted in a more florid infection of the ganglia and spinal cord which led to a higher incidence of latency. Conclusions from this and another study (Kapoor *et. al.*, 1982b) suggested that neutralising antibody plays a role in restricting the spread of HSV to the nervous system whilst CMI is important in clearing virus from the site of infection.

The mouse models of HSV disease have also produced data which outline the roles of T cell sub-sets in the clearance of virus from various tissues. Nash & Gell (1983) showed that activated $CD4^+$ T cells could transfer DTH to mice which could then clear infectious virus from epidermal surfaces. These cells produce IFNγ and have the general properties of Th1 cells (Seid *et. al.*, 1987). The mechanism by which

IFNγ producing CD4$^+$ T cells cleared virus from the tissues was discussed by Nash & Cambouropoulos (1993) who suggested that IFNγ induced the recruitment of monocytes from the blood which accumulated at the site of infection and became activated macrophages under the influence of IFNγ. These were then able to clear virus via the production of TNFα, IFNα/β, arginase and cationic proteins.

The role of CD8$^+$ T cells, particularly CTL, in the clearance of HSV from infected tissues is controversial. Adoptive transfer of HSV specific CTL protects mice against disease (Larsen *et. al.,* 1983, Sethi *et. al.,* 1983), however, the relevance of *in vitro* matured or cloned CTL to the *in vivo* situation is questionable. Nash *et. al.,* (1987) showed that CD8$^+$ T cells contribute to the control of infectious HSV in the neurons, however, the lack of any neural cell damage caused by these cells suggests that cytolysis was not a major mechanism in controlling virus levels. A study by Smith *et. al.,* (1994) specifically designed to elucidate the roles of CD4$^+$ and CD8$^+$ T cells in the clearance of HSV from the footpad of C57BL/6 (B6) mice showed that depletion of both subsets resulted in failure to clear virus from the skin. Depletion of either CD4$^+$ or CD8$^+$ T cell subsets, using monoclonal antibodies, had little effect on the amount of virus isolated. Interestingly, depletion of either subset abolished CD8$^+$ CTL activity from the lymph nodes and the spleen, confirming that, in B6 mice, the anti HSV CTL response was CD4$^+$ T cell dependent (Jennings et. al., 1991). These data suggest that the mechanism of virus clearance was common between both subsets and, whilst cytolytic mechanisms active in the skin, could not be discounted, IFNγ dependent mechanisms were implicated as mice injected with neutralising anti-IFNγ antibody

failed to clear virus from the footpad. It is now well documented that $CD8^+$ T cells as well as $CD4^+$ T cells are able to produce relatively high levels of cytokines, particularly IFNγ (Fong & Mossman, 1990. Taguchi *et. al.*, 1990).

The ultimate goal of many viral immunologists is to identify the minimal epitopes present in virus proteins which are recognised by specific antiviral T cells. This can be achieved on a number of levels, for example, T cells can by distinguished as type specific or cross-reactive (Doherty *et. al.*, 1977), the broad classes of proteins recognised by antiviral T cells can be determined (Tigges *et. al.*, 1992), the specific virus proteins which elicit a T cell response can be identified, and ultimately, the minimal T cell epitopes within a protein can be isolated (Bonneau *et. al.*, 1993). Analysis of these responses in in-bred mouse strains is the norm in this type of study although it is likely that mouse strain-specific epitopes will be identified.

Initial studies identified HSV glycoproteins as being important in stimulating cell-mediated immunity and in particular, CTL (Lawman *et. al.*, 1980a, Carter *et. al.*, 1981). The use of T cell cloning methods indicated that the majority of HSV specific CTL clones from C57/Bl mice recognised the glycoprotein gC (Glorioso *et. al.*, 1985). Rosenthal *et. al.* (1987) showed that HSV-specific CTL from CBA mice recognised gC but not gB, gD or gE. Studies using recombinant vaccinia viruses expressing isolated glycoproteins from HSV (Mester & Rouse, 1991) have shown that a gC expressing recombinant generated gC specific CTL in b haplotype mice but not in k or d haplotype mice. In the same study recombinant vaccinia viruses expressing

gB or gD were unable to consistently generate CTL activity. However, expression of gB and gD in mouse L cells and subsequent immunisation of mice with these cell lines did elicit protective responses (Blacklaws *et. al.*, 1987). Vaccination of Balb/c mice with recombinant baculoviruses expressing seven different HSV-1 glycoproteins showed that protection against lethal intraperitoneal or ocular challenge was stimulated using gB, gC, gD, or gI recombinants (Ghiasi *et. al.*, 1994). DTH responses in these mice were highest on day 3 using gG, gH or gE recombinants and on day 6 using gC, gE and gI recombinants. The same five glycoproteins expressed in recombinant baculoviruses (*i.e.* gC, gE, gB, gD and gI) which stimulated protection also elicited CTL activity, although the anti gE response only stimulated CTL measurable at the 100:1 effector:target ratio. The minimal epitope on gB recognised by HSV-1/2 cross-reactive CTL derived from C57Bl/6 (H-2^b) mice was determined by Bonneau *et. al.* (1993). In this study, CTL clones were isolated which recognised an epitope consisting of residues 498-505 from HSV gB. Immunisation of mice with the free synthetic peptide resulted in the generation of $CD8^+$ CTL in lymph nodes. In a further study by the same group (Salvucci *et. al.*, 1995) HSV-1 specific CTL clones were isolated from H-$2K^b$ mice which recognised an epitope on the HSV-1 non-structural ribonucleotide reductase large subunit (ICP6) consisting of residues 822-829.

Other non-structural viral proteins, for example the immediate early (IE) proteins, have been investigated as target antigens for CTL in the mouse. The HSV IE proteins ICP4 and ICP27 act as target antigens in H-2^b and H-2^d mice respectively (Martin *et.*

al., 1990, Banks *et. al.*, 1991). ICP27 expressed in a recombinant vaccinia virus stimulated HSV-1 specific CTL activity and also protected mice from a lethal challenge with HSV-1 and HSV-2 (Banks *et. al.*, 1991). Two minimal T cell epitopes were generated from ICP27 (Banks *et. al.*, 1993). Both peptides, residues 322-332 and 448-456, sensitised target cells for recognition by HSV-specific CTL and generated primary CTL *in vitro*. The advantage of using non-structural proteins as CTL epitopes rather than viral glycoproteins is that the virus may be eliminated before progeny virus is produced.

In summary, the recognition of antigens by HSV-specific T cells in mice depends on the mouse strain being studied, the method of production of the antigen and the route of infection of any subsequent challenge. However, it does seem likely that T cells recognise a spectrum of HSV proteins during an active infection, including the non-structural proteins and a number of glycoproteins.

1.2.4.7. Immunity to HSV in humans

In recent years, research on the murine immune response to HSV has outweighed research on the human response. Studies in humans have concentrated on the CTL responses to HSV, although some papers outline the proliferative and cytokine responses, especially to viral glycoproteins. For example, Torseth *et. al.* (1987) showed that combinations of purified glycoproteins stimulated *in vitro* lymphocyte proliferation and cytokine production as efficiently as whole HSV preparations. Zarling *et. al.* (1986) demonstrated that purified HSV gB-1 and gD-1 stimulated

proliferation, IL-2 production and CTL activity in PBMC from HSV sero-positive individuals. Damhof *et. al.*, (1993) used overlapping peptides derived from gD to stimulate proliferation of PBMC from 10 healthy HSV sero-positive individuals. The results from this study suggested that there was no correlation between sero-positivity and proliferative response and that there were no immuno-dominant epitopes within the gD molecule, at least as far as the proliferative response was concerned. Antigen-specific proliferation assays were useful in the detection of immune activity in sites other than the blood, for example, Mathiesen *et. al.* (1990) demonstrated antibody producing and proliferative lymphocytes in peripheral blood and cerebro-spinal fluid from patients with HSV encephalitis.

HSV-1/HSV-2 cross-reactive and HSV-1 type common T cell clones which were both cytotoxic and able to proliferate *in vitro* were isolated in a study by Yasukawa & Zarling (1984). These T cells were $CD4^{+}CD8^{-}$ and also had the capacity to produce IL-2. The multi functional nature of these CTL clones and the class II MHC restriction was probably due to the use of UV inactivated virus in the cloning procedure. The stimulation of anti-HSV $CD4^{+}CD8^{-}$ CTL using UV inactivated virus was confirmed (Schmid, 1988 and Torpley *et. al.*, 1989) although the contention by Schmid that the human CTL response to HSV-1 was mainly restricted by class II MHC fuelled debate. It is now clear that, whilst induction of CTL *in vitro* with inactivated HSV results in $CD4^{+}CD8^{-}$ CTL, the more relevant induction using infected cells results in the stimulation of, mainly, $CD4^{-}CD8^{+}$ CTL (Yasukawa *et. al.*, 1989 and Maccario *et. al.*, 1992). However, the isolation of T cell clones derived

directly from HSV-2 lesions and stimulated *in vitro* in cultures containing no HSV antigen resulted in $CD4^{+}CD8^{-}$ clones exhibiting proliferative or cytotoxic activity against numerous epitopes within gB, gC, gD and VP16 (Koelle *et. al.*,1994). Tigges *et. al.* (1992) demonstrated that seven $CD4^{-}CD8^{+}$ CTL clones recognised five different patterns of HSV proteins with one clone recognising HSV IE protein and six recognising virion proteins amongst them gD but not gB or VP16 (the tegument protein).

1.2.4.8. Immunity to other herpesviruses

As outlined above, the immune response to HSV has been widely studied. Significant progress has also been made in the study of the immune response to other herpesviruses.

T cell-mediated immunity is important in maintaining the latent state of VZV as shown by the occurrence of VZV reactivations in elderly patients whose T cell, but not antibody, levels have been reduced as a result of ageing (Hayward & Herberger, 1987). The T cell proliferative response of PBMC to VZV antigens has been used to correlate virus-specific CMI with disease outcome (Arvin, 1992). For example, immunodeficient children who fail to acquire a VZV proliferative response develop progressive disseminated disease, whereas, healthy children who develop a detectable response after the appearance of a varicella rash develop a mild primary VZV disease. Studies on the T cell response to VZV have been stimulated by the fact that vaccination with the live attenuated Oka strain of VZV occasionally results in clinical

symptoms of herpes zoster in vaccinated individuals as a result of reactivation of the vaccine strain (Lawrence *et. al.*, 1988). The need to replace the live attenuated vaccine has led to numerous studies to determine which VZV proteins stimulate CMI. In T cell proliferation assays, gpI, gpIII and p170 (the product of the VZV IE gene) are recognised (Arvin *et. al.*, 1986). Hayward showed that gpII and gpIV synthetic peptides stimulated $CD4^+$ cells to proliferate (Hayward, 1990). Natural infection also elicits CMI against gpII and gpV. Responses to all of these proteins lasts for years after primary infection (Arvin, 1992). Both $CD4^+$ and $CD8^+$ CTL have been demonstrated after VZV infection (Arvin *et. al.*, 1991). These CTL recognise gpI, gpIV, gpV and p170 (Huang *et. al.*, 1992).

There is little correlation between anti-PRV antibody titre and protection against infection with PRV (Martin *et. al.*, 1986, Matsuda-Tsuchida *et. al.*, 1992). Anti-PRV CTL activity has been demonstrated in pigs and in a mouse model of PRV disease (Zuckermann *et. al.*, 1990). In this study, $CD4^-CD8^+$ CTL were induced using PRV and assayed on target cells infected with mutant PRV viruses lacking one of the non-essential glycoproteins gI/gp63, gX or gIII. The results show that lysis of targets lacking gIII was significantly reduced when compared with targets infected with the wild type virus or viruses lacking the other glycoproteins. This result indicated that the gIII molecule was recognised by anti-PRV CTL.

CTL against the bovine respiratory virus Bovine herpesvirus-1 (BHV-1) were first demonstrated by Campos and Rossi (Campos & Rossi, 1986). It was shown that

different BHV-1 glycoproteins were recognised by CTL depending on the method of *in vitro* stimulation (Denis *et. al.*, 1993). In this study, target cells expressing gIV were lysed when CTL were induced using fixed infected fibroblasts whilst gIII expressing target cells were predominantly lysed when CTL were induced with UV inactivated virus. This result suggests that the antigenic specificity of CTL detected *in vitro* could be influenced by the culture system used.

Anti CMV antibodies are not important in protection of patients from CMV infection as seropositive allograft recipients are still at risk of reactivation or super-infection with CMV (Pasternack *et. al.*, 1990). Studies of murine CMV (mCMV) have shown that the dominant antigens recognised by anti mCMV CTL were the products of the IE genes (Reddehase & Koszinowski, 1984). This result indicates that the search for immunodominant antigens should not be restricted to the structural proteins.

1.2.4.9. Evasion of the immune response

Many viruses have evolved mechanisms by which they evade the immune responses of the host. Antigenic drift, the gradual changes in the antigenicity of the HA molecule in the influenza A viruses, is probably the best studied escape mechanism (Webster *et. al.*, 1982). Antigenic drift is a result of pre-existing antibody guiding the evolution of new influenza strains. A similar type of escape from CTL recognition has been demonstrated *in vitro* for lymphocytic choriomeningitis virus (Aebischer *et. al.*, 1991).

Human immunodeficiency virus (HIV) employs numerous mechanisms in order to evade the host immune response. Firstly, it infects cells of the immune system which may prevent them from mounting a relevant response. Secondly, sequence variations within the CTL epitopes of HIV proteins mean that the virus can replicate in the face of a vigorous CTL response (Koup, 1994). Recent studies have shown that HIV is able to stimulate an oligoclonal B cell response which predominates in the face of HIV mutation. The resultant, altered virus is then able to escape neutralisation by the oligoclonal antibody (Kohler *et. al.*, 1995).

Retroviruses also affect the host immune response by inducing a state of hyporesponsiveness in T lymphocytes (Orosz *et. al.*, 1985). A similar phenomenon has also been reported in association with infection with measles virus (Lucas, 1977), herpes simplex virus (Plaeger-Marshal & Smith, 1978) and EHV-1 (Hannant *et. al.*, 1991). Persistence of herpesviruses within the host is facilitated by latency (Ahmed, 1990). Latency allows herpesviruses to avoid immune recognition until the host immune system is compromised at which point reactivation occurs.

Viral structural proteins may be directly immuno-suppressive as Newman *et.al.* (1991) reported for equine infectious anaemia virus. Certain viruses contain genes the products of which, interfere with immune processes. Human CMV expresses a homologue of MHC class I heavy chain which can bind to ß2M preventing the maturation of cellular class I molecules and subsequent recognition by CTL (Browne *et. al.*, 1990). Infection of human dermal fibroblasts with HSV has also been shown

to result in the down regulation of the cell surface MHC class I (Hill *et. al.*, 1994). Epstein-Barr virus has the potential to suppress the host's immune system due to the presence in it's genome of an IL10-like gene; this cytokine is capable of inhibiting Th1 T cell responses (Moore *et. al.*, 1990). A similar gene is present in the equine herpesvirus 2 genome (Rode *et. al.*, 1993) but this has not been described for EHV-1. Ward *et. al.* (1990) reported that the transient depression of DTH and mitogen induced proliferation of human PBMC after measles virus infection (or vaccination with a live vaccine) was related to the preferential activation of Th2 cells producing high levels of IL4. This preferential activation of either Th1 or Th2 cells may have the effect of skewing the immune response away from an effective response. This also may occur after infection with HIV (Clerici & Shearer, 1993).

1.3. Equine herpesvirus types 1 and 4

EHV-1 and EHV-4 are primarily transmitted via inhalation of infected particles and acute infections with both viruses causes respiratory disease. Infection with EHV-1 may also result in abortion and neurological disease (Allen & Bryans, 1986). Both EHV-1 and EHV-4 have the capacity to become latent, probably residing in the lymphoreticular system (Welch *et. al.*, 1992). Together, these viruses cause considerable, worldwide economic loss within the racing and breeding industries.

1.3.1. Pathogenesis

Infection of young, immunologically naive, horses with either EHV-1 or EHV-4 is clinically manifest by a biphasic pyrexic response and a serous nasal discharge which

rapidly becomes mucopurulent (Allen & Bryans, 1986). Anorexia and depression, coughing, enlarged intermandibular and retropharyngeal lymph nodes and congestion of the nasal mucosa may also occur (Bryans, 1964, 1980. Coggins, 1979). In older, immunologically experienced animals infection may give rise to mild or inapparent clinical symptoms (Bryans, 1981). The majority of studies on the pathogenesis of EHV infection have looked at EHV-1. Strains of EHV-4 are usually cleared from the respiratory tract within 7 to 20 days after primary infection and within 2 to 7 days following subsequent infections (Mumford, 1994). Infection with EHV-4 in most instances is limited to the respiratory tract because the virus does not usually cause a cell associated viraemia (Burrows & Goodridge, 1972. Edington *et. al.*, 1986). EHV-4 is, therefore, often perceived as a less severe, primarily respiratory, infection (Coignoul *et. al.*, 1984) although in very rare cases it may cause viraemia and abortion.

After inhalation of infected particles EHV-1 replicates in the epithelial lining of the respiratory tract and spreads, intracellularly. Patel *et. al.* (1982) showed that infectious virus and viral antigens were present in the nasal mucosa, lungs and retropharyngeal lymph nodes 4 days after infection and in the submandibular lymph nodes 8 days after infection of a naive foal with EHV-1. Some EHV-1 strains then infect vascular endothelial cells (Patel *et. al.*, 1982. Edington *et. al.*, 1986) and subsequently cause a cell-associated viraemia, abortion and/or paresis (Edington *et. al.*, 1991). However, some other EHV-1 isolates do not replicate in endothelial cells. Kydd *et. al.* (1994a & 1994b) showed, using both virus isolation and an indirect

immunoperoxidase technique to detect viral antigens, that EHV-1 was present in the lymph nodes draining the respiratory tract of horses within 12 hours after infection with a known abortogenic strain of EHV-1 (EHV-1/Ab4) which was originally isolated from a paraplegic gelding (Edington *et. al.*, 1986). A model for the progression of this strain of EHV-1 from the respiratory tract was suggested (Kydd *et. al.*, 1994b) in which a productive infection of the epithelial lining of the nasopharynx results in spread of the virus through susceptible cells, including leucocytes, resulting in the infection of vascular or lymphatic endothelium and the establishment of viraemia. Edington *et. al.* (1986) showed that endothelial cell infection, followed by thrombus formation, associated haemorrhage and ischaemic anoxia in the central nervous system occurred in animals infected with EHV-1 strain Ab4. Endothelial cell infection by EHV-1 was also implicated in the pathogenesis of EHV-1 strain A183-induced abortion (Edington *et. al.*, 1991). The endotheliotropism of EHV-1 has been demonstrated by immunohistochemical investigation of a natural outbreak of EHV-1 abortion and neurological disease (Whitwell & Blunden, 1992).

The mechanisms involved in the abortion of the equine foetus due to EHV-1 remain elusive although recent studies have shown that infection of endothelial cells is associated with endometrial vasculitis and thrombosis (Smith *et. al.*, 1993). In the model suggested by Smith *et. al.* the degree of thrombosis determines the outcome of pregnancy. Widespread thrombus formation and severe endometrial infarction results in the early expulsion of a virus-negative foetus; limited infarction results in the expulsion of a virus-positive foetus and no infarction results in a normal foetus. The

occurrence of virus-negative aborted foetuses prior to a major EHV-1 induced abortion storm has been documented (Mumford *et. al.*, 1987). Such foetuses are also produced in a proportion of animals after experimental challenge of pregnant pony mares with EHV-1 strain Ab4 (Smith *et. al.*, 1992). In these cases, abortion results from virus-induced immunopathology at the maternal side of the materno-foetal interface and consequent rapid expulsion of the foetus, before infection becomes established.

1.3.2. The antibody response

Doll (1961) showed that horses become susceptible to re-infection with a homologous strain of EHV-1 3-5 months after a primary infection. This re-infection can take place despite the presence of high levels of virus neutralising antibody in the serum (Mumford *et. al.*, 1987). Notwithstanding the apparent insignificance of antibody to protection against EHV-1 infection, a considerable amount of work has been carried out on characterising the antibody response to EHV-1/-4 in conventionally reared (Coignoul *et. al.*, 1984. Stokes *et. al.*, 1991. Dolby *et. al.*, 1995) and SPF (Gibson *et. al.*, 1992a) horses. After EHV-1 or EHV-4 infection of naive horses, antibody, as measured using a complement fixation (CF) or virus neutralisation (VN) assay, appears in the serum after approximately 6-8 days (Stokes *et. al*, 1991. Gibson *et. al.*, 1992a). VN antibody is relatively long lived persisting for months after infection, particularly in older animals. CF antibody becomes undetectable relatively quickly after infection (Thomson *et. al.*, 1976b) making this a reliable marker for recent exposure to the virus. After secondary infection an anamnestic response occurs.

The production of a wide range of anti EHV-1 and EHV-4 monoclonal antibodies has allowed improvements in the diagnosis (Dutta *et. al.*, 1983. Allen & Bryans, 1986. Sinclair & Mumford, 1992) and epzootiological (Matsumura *et. al.*, 1992) aspects of EHV infection. However, the cross-reactive nature of most EHV-1/-4 glycoproteins limits the usefulness of these reagents (Crabb & Studdert, 1990. Crabb *et. al.*, 1991. Sinclair, 1991). Recently, an EHV-4 type specific glycoprotein (gG) has been identified (Crabb *et. al.*, 1992); the EHV-1 counterpart also contains type specific epitopes (Crabb & Studdert, 1993).

One aspect of the antibody response to EHV-1 or EHV-4 which has not been extensively studied, yet holds great potential to control natural infections with both viruses is that of mucosal immunity. The fact that both EHV-1 and EHV-4 infect mucosal surfaces initially suggests that infection could be blocked via the stimulation of mucosal antibody. This has been achieved experimentally with BHV-1 (Israel *et. al.*, 1992) using mucosal vaccination with inactivated antigens. Nasopharyngeal antibody was stimulated in horses vaccinated with EHV-1 in Freund's complete adjuvant (Dolby *et. al.*, 1995). These horses were protected from experimental infection. More work needs to be done in this area.

1.3.3. Cell-mediated immunity

Until recently, the study of CMI responses of horses to EHV-1 or EHV-4 concentrated on the *in vitro* proliferation of isolated equine PBMC (Thomson & Mumford, 1977. Dutta *et. al.*, 1980). Valuable data were obtained from these early studies; for

example, Dutta & Campbell (1977) showed that some mares with high VN antibody titres and proliferative responses to inactivated EHV-1 antigen aborted after infection whilst others did not. This suggested that neither of these immune functions were reliable markers of protection against infection with EHV-1.

Thomson & Mumford (1977) produced the first evidence that equine mitogen-stimulated PBMC were able to support a productive infection with EHV-1 (strain RAC-H). These workers also produced evidence that infection of horses with certain strains of EHV-1 suppressed *in vitro* lymphocyte functions (Thomson & Mumford, 1977. Dutta *et. al.*, 1980). Hannant *et. al.* (1991) showed that the *in vitro* response of equine PBMC to the mitogen PHA was suppressed in 6 ponies after infection with EHV-1 (strain V592), whereas antigen-specific proliferative responses in these animals were not suppressed. This immunosuppression induced by infection with EHV-1 may predispose horses to more severe, unrelated infections.

The pace of research into the CMI response to EHV-1/-4 has been accelerated by the provision of monoclonal antibodies specific for equine lymphocyte cell surface markers (Kydd *et. al.*, 1994c). These monoclonal antibodies are invaluable in studies of the phenotype of responding cells.

A mouse model of EHV-1 infection has been developed (Awan *et. al.*, 1990) in order to study the pathogenesis of and immune response against EHV-1. Despite major differences to EHV-1 infection of the horse (low antibody titres, failure to induce VN

antibody and relatively long-lived immunity stimulated by infection), studies have demonstrated that DTH responses develop in infected mice (Azmi & Field, 1993a). These were of a higher magnitude in intranasally infected than in intravenously inoculated mice, although intravenous inoculation produced a higher antibody response. Further studies showed that EHV-4 was apathogenic in BALB/c mice although exposure to EHV-4 conferred some protection against EHV-1 infection (a reduction in the level of virus excreted from the respiratory tract). *In vivo* depletion of $CD4^+$ or $CD8^+$ cells from EHV-4 infected (EHV-1 immune) mice prior to infection with EHV-1 showed that only depletion of $CD8^+$ cells resulted in a reduction of the protective effect. Although the protective immune response resulted in a reduction in the amount of virus isolated from the tissues, this did not necessarily, correlate with a reduction in mortality (Azmi & Field, 1993b).

Recently, Allen *et. al.* (1995) have developed methods for the characterisation of the anti EHV-1 CTL response of horses after infection. This will prove valuable in the evaluation of the role of CTL in protection against EHV-1 infection.

1.3.4. Vaccination

Although effective management of horses at stud can go some way to reducing the incidence of EHV infections, vaccination of the horse population with an effective vaccine is the only practical way to prevent EHV disease.

The observation that mares rarely abort as a result of EHV-1 infection in consecutive seasons prompted a large scale planned infection of mares in Kentucky. The virus used was a partially attenuated, hamster adapted strain of EHV-1 (Rac-H). Rates of abortion were reduced from 30% in the untreated population to 1.5% in treated animals as a result of the planned infection (Doll & Bryans, 1963).

Two subsequent commercially produced live attenuated vaccines based on the Rac-H strain were produced. Rhinomune (Beckenhauser & Bass, 1973) was further passaged in equine cell culture and Prevaccinol (Mayr, 1970) was further passaged in pig kidney cells. Both of these live attenuated vaccines were shown to cause a low incidence of abortion. Following field immunisation studies with Rhinomune, a virus identical to the vaccine strain was isolated from aborted foetuses (Allen *et. al.*, 1983). Similarly the vaccine virus was isolated from aborted foetuses after intra-uterine inoculation (Burrows & Goodridge, 1978) with the vaccine Prevaccinol.

As it is clearly difficult to produce a safe and effective live attenuated vaccine for control of EHV-1, attempts have been made to produce killed or subunit vaccines. A commercially licensed, killed vaccine, Pneumabort K, was introduced in 1980. This contained EHV-1 strain A183 grown in equine cell culture, inactivated with formaldehyde and combined with an oil adjuvant. Although Pneumabort K failed to prevent abortion in some individual animals (Burrows *et. al.*, 1984) it's widespread use in the Kentucky area has more than halved the number of abortions per 1000 mares.

The techniques are now available with which to produce subunit vaccines although none have been licensed for use as yet. Potential vaccines based on the production of mutant viruses lacking genes that determine virulence, large amounts of isolated viral antigens (*e.g.* glycoproteins) and hybrid viruses incorporating genes from EHV-1 and EHV-4 have all attracted research effort recently.

As mentioned above, perhaps the best candidate for an effective and safe EHV-1 vaccine is a mucosally applied preparation combining viral proteins with adjuvants which target the antigens to lymphoid tissue. This type of vaccine is still some way from even being experimentally produced.

2. Target cell evaluation

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2.1. Introduction

As outlined in the previous chapter, CTL may be generated *in vivo* by herpesvirus infection (Rouse & Horohov. 1984) and also, under certain circumstances, using isolated viral proteins (Bachmann *et. al.*, 1994). On secondary culture of lymphoid cells *in vitro* with specific antigen, precursor cytotoxic T lymphocytes (CTLp) are induced to develop into effector CTL capable of killing virally infected cells in an antigen specific, genetically restricted manner. The measurement of effector CTL involves providing optimum conditions under which effector and target cells recognise each other via contact between the T cell receptor and MHC/peptide complex. A stable conjugate, strengthened by non-specific attachments between numerous molecules (e.g. LFA-1 to ICAM-1, CD2 to LFA-3 and CD8 to MHC class I), is formed which leads to the delivery of a lethal hit to the target cell. The mechanisms involved in the delivery of the lethal hit are outlined in chapter 1.

The quantitation of this process *in vitro* is usually carried out by means of the release of radioactive label from target cells - the ^{51}Chromium release assay. As applied to viral infections, the assay is performed by mixing virus-infected or peptide pulsed, autologous, ^{51}Cr labelled target cells with putative effector CTL populations (Zweerink *et. al.*, 1977). After a period of time, the cells are removed by centrifugation and the amount of ^{51}Cr released by the target cells is estimated by counting the radioactivity in cell-free supernatants in a gamma counter. The released radioactivity (i.e. ^{51}Cr) must be considered to be proportional to the amount of target cell lysis rather than an exact measure of it (Martz, 1993). Other factors affecting the

release of ^{51}Cr are the type of target cell used, the history of the target cell (*i.e.* the labelling procedure, time since labelling *etc*), the chemical environment prior to lysis and the time interval between lysis and separation of soluble from insoluble ^{51}Cr.

When labelled with ^{51}Cr a target cell will spontaneously release between 1% and 10% of the total incorporated radioactivity per hour (Martz, 1993). This leakage is mainly due to cell damage during preparation although isotope may also be slowly released from some viable cells. This effectively limits the duration of cytotoxicity assays to a maximum of about 18 hours. However, the majority of ^{51}Cr release assays used in viral systems are performed over a period of between 4 and 6 hours. Control measurements are a necessary part of ^{51}Cr release assays. Spontaneous release of label over the period of the assay is measured by culturing target cells without effector cells. This should always be less than 35% of the total releasable label as spontaneous release exceeding 35% may not represent lysis at all, but changes in the chemical association of the label with cellular debris (Martz, 1993). Measurement of total releasable label (an assessment of 100% lysis) is carried out by lysing the target cells using either detergents (e.g. triton x100 or NP40) or by freezing and thawing the target cells. Either of these two measurements are used to calculate the % specific release of label. Detergent lysis often produces higher total releasable values than freezing and thawing presumably due to the proteins being released from membrane fragments.

Other methods for quantitation of cytolysis do exist, for example the amount of target

cell killing in a culture can be assessed by counting the number of dead cells which are able to take up a dye reagent e.g. trypan blue or eosin. These counting methods, although used extensively in early cytotoxicity studies (Takasugi & Klein, 1970) are now recognised as being subjective, tedious and imprecise.

However, there are disadvantages in using ^{51}Cr release assays to measure CTL activity. They are unable to provide precise measurements of high levels of killing because of the crude means of assessing 100% lysis. However, when used under optimum conditions the major advantages of ^{51}Cr as a target cell label are that it is relatively non-toxic to the target cells, is not significantly reutilised by effector cells on release from target cells and allows the processing of large numbers of effector populations in one assay (Martz, 1993).

A wide range of target cell types have been used to quantify CTL activity (e.g. hepatocytes by Ayres *et. al.*, 1991, kidney cells by Mc Guire *et. al.*, 1994, macrophages by Kasper *et. al.*, 1992). In theory, any cell type which expresses the appropriate MHC can be used as a target cell for CTL. However, the limitations of the assay mean that cells which spontaneously release high levels of chromium and those which cannot present viral peptides to active CTL are unsuitable. The target cell type should also be easy to isolate and manipulate *in vitro*. The popularity of murine models for human and other animal diseases, means that murine MHC typed fibroblastic cell lines are increasingly being used as targets (Shultz *et. al.*, 1991). In many human diseases CTL may be measured using Epstein-Barr virus transformed B

cells as targets (Green *et. al.*, 1993). In order to measure anti EHV-1 specific CTL, the target cell must also be permissive to infection with the virus. Moreover, it is desirable for the majority of the permissive target cells in a population to be infected. The infection of the majority of the target cells means that viral peptides are likely to be presented in the context of MHC molecules and the target cells are therefore likely to be recognised by CTL.

In summary, the critical properties of a useful CTL target are, ease of isolation and handling, retention of the majority of the label (more than 65%) over the period of the CTL assay and the presentation of viral peptides within MHC on the surface of the majority of the permissive cells in the target cell population.

This chapter outlines experiments carried out in order to evaluate a range of equine cells as potential targets for use in an EHV-1 specific CTL assay. Cells were isolated from peritoneal fluid, vascular endothelium and peripheral blood mononuclear cell (PBMC) preparations and tested for their permissiveness to EHV-1 infection, and ability to retain ^{51}Cr label.

2.2. Materials and Methods

2.2.1. Isolation of equine peritoneal macrophages

Equine peritoneal fluid was collected by mid-line puncture of the peritoneal cavity of adult mares with a large gauge needle under local anaesthesia. The fluid was collected into preservative-free heparin, centrifuged, and the cells were washed in

Hank's balanced salts solution (HBSS). Peritoneal macrophages (PM) were isolated from the polymorphonuclear leucocytes in the fluid by adhering them to the plastic surface of a sterile petri dish for 60 minutes at 37°C. The non-adherent cells were removed from the dish which was washed in HBSS. The PM were scraped from the dish into complete RPMI (cRPMI) using a rubber policeman.

2.2.2. Isolation of equine vascular endothelial cells

Equine vascular endothelial cells were isolated at postmortem from the carotid artery using the method of Lamar *et. al.* (1986). The isolated cells were maintained in T150 tissue culture flasks and passaged when confluent. They were periodically tested for the expression of the endothelial cell marker von Willibrands factor using a specific monoclonal antibody (Dako) in the immunoperoxidase test described in 2.2.6.

2.2.3. Production of *in vitro* matured PBMC

Blood was collected from horses by jugular venipuncture into preservative-free heparin (300 units per 20ml tube) using evacuated blood tubes (Vacutainer, Becton Dickinson). The blood was kept at room temperature for approximately 30 min until a distinct leucocyte rich plasma layer could be seen. The leukocyte rich plasma was gently layered onto 3ml of Ficoll Hypaque (Pharmacia) in a 10ml conical centrifuge tube so that a distinct interface could be seen between the plasma and the separation medium. The tubes were centrifuged at 400g for 30 min at 22°C, with the brake off. The resulting band between the plasma and Ficoll layers contained the PBMC fraction. The band was removed and the cells were washed twice in HBSS (see

appendix A for formulation). The PBMC were resuspended at a concentration of 1×10^7 cells ml^{-1} in cRPMI containing 20% normal horse serum. The cells were incubated at 37°C for 21 days by which time large macrophage-like cells constituted about 50% of the live cells. They were then washed in HBSS and resuspended in cRPMI.

2.2.4. Production of lymphoblasts

Equine PBMC were isolated using Ficoll Hypaque as described above. The resultant PBMC were then cultured with mitogen in order to produce maximum proliferation. The optimal concentration for each mitogen used was as follows:

Mitogen	Optimum concentration
Concanavalin A	5.0µg/ml
Phytohaemagglutinin	2.5µg/ml
Pokeweed mitogen	2.5µg/ml

Maximal proliferation was achieved with a culture time of 72 hours. In practice, incubation times were varied in order to accommodate any further treatment of the target cells depending on the experiment e.g. infection or chromium labelling.

2.2.5. Antibody binding assays

Equine cells were isolated and resuspended to the required concentration in cold HBSS. Cells were dispensed into sterile plastic removawells (Dynatech) at between 1

and 2×10^5 cells/well. The plates were centrifuged for 3 min at 180g to pellet the cells, the supernatant was removed using a vacuum pump and an optimal dilution of the primary antibody in cRPMI was added. Wells were incubated for 1 hour at 4°C in order to avoid antibody capping. The wells were washed 3 times with ice cold cRPMI by centrifugation and removal of the supernatants. If the primary antibody was from a rabbit then 1×10^5 counts per minute of ^{125}Iodine-labelled *Staphylococcus aureus* Protein A (Prot A) was added to each well. If the primary antibody was from any other species, then an appropriate dilution of rabbit anti-species immunoglobulin was added, incubated as above for 1 hour, washed three times and then the Prot A was added. Wells were incubated for 1 hour at 4°C. The wells were washed 3 times with ice cold cRPMI leaving the pellet dry after the third wash. Individual wells were counted in a γ counter (Philips). Controls were mock-infected cells incubated with the test antibodies.

2.2.6. Immunoperoxidase staining of cytospin preparations

This method was used to demonstrate the proportion of a population of cells which expressed viral antigen after infection. This was more useful than the results of the antibody binding assay which only provided an estimate of the presence of antigen expression.

Cytospins were prepared using 2 drops of cells from a 2×10^6 cells ml^{-1} suspension and preparations were air dried for 30 minutes and then fixed in acetone for 10 minutes. Fixed cytospin preparations were stored at -20°C. Before staining, the cytospins were

equilibrated at room temperature for 10 minutes. The disks of cellular material were ringed with a water resistant pen (Dakopen, Dako Ltd.) in order to contain the antibody solutions.

All incubations were carried out at room temperature in a humidified box. The cytospin disks were flooded with 150μl of primary antibody (e.g. rabbit anti EHV-1 which had been extensively absorbed against equine PBMC) diluted to an optimal concentration (1/500) in Tris buffered saline (TBS) containing 2% bovine serum albumin (BSA, Sigma Chemical Co, Poole, Dorset). They were incubated for 45 min, washed in fresh TBS for 5 min and flooded with 150μl of a 1/200 dilution of secondary antibody peroxidase conjugate (e.g. goat anti rabbit peroxidase conjugate, Dako Ltd.) in TBS + 2% BSA. After a further 45 min incubation, the slides were washed in fresh TBS and flooded with 150μl of substrate mixture (22.5ml 0.85% NaCl, 2.5ml fresh 0.2M sodium acetate at pH5.5, 200μl of a 0.3% hydrogen peroxide solution in distilled water and 3ml 3-Amino-9-Ethylcarbazole in dimethyl sulphoxide, all reagents from Sigma Chemical Co, Poole, Dorset). After incubation for 45 min the slides were washed in fresh TBS and counterstained for 2 min in Mayer's haemalum (Raymond A Lamb, London). They were washed in running water for 5 min and mounted in a glycerol/gelatin mountant (Sigma).

2.2.7. ^{51}Chromium labelling of cells

The methods of labelling equine cells with ^{51}Cr varied depending on the cell type used. For the majority of the preliminary studies, cells were labelled for 60 min.

The ^{51}Cr labelling procedure was based on that used by O'Brien (1992). Briefly, cells were pelleted and resuspended in approximately 200μl of cRPMI containing 10% foetal bovine serum. This medium was found to give the highest uptake of ^{51}Cr and showed the lowest spontaneous release of label in a preliminary experiment when cells from the stable rabbit kidney cell line RK13 were labelled with ^{51}Cr. Approximately 100μCi of ^{51}Cr* (Amersham International PLC) per $5x10^6$ cells were added to the cell suspension and the cells were incubated for 60 min at 37°C in an atmosphere of 5% CO_2 in air. The labelled cells were then washed twice in 10ml HBSS and once in 10ml cRPMI. They were resuspended to a concentration of $1x10^6 ml^{-1}$ in cRPMI and 100μl of the cell suspension was counted in a γ counter in order to derive a figure for the total incorporated label, and hence the amount of label per cell. Labelled cells were then plated in round bottomed 96 well plates containing either cRPMI or the detergent triton x100 (2.5% in PBS) in order to derive figures for percentage release of label over time for each cell type.

The method for the spontaneous/total release determination was as follows: Samples of the labelled target cells were dispensed in 100 μl at a number of concentrations in triplicate in 96 well plates (U shaped wells). The cell number per well was usually between $1x10^4$ and $1x10^5$. An equal volume (100μl) of cRPMI was added to one set of wells and an equal volume of triton x100 was added to the other. Plates were centrifuged at 180g for 2 min. Volumes of 100μl of the supernatants from the cRPMI wells (spontaneous release) and the triton wells (total release) were counted in a γ counter. The % spontaneous release of chromium from the culture was derived using

* 100-500 mCi/mg Cr.
10 - 35 mCi/ml.

the formula: **cpm spontaneous/cpm total x 100.**

2.3. Results

2.3.1. Evaluation of labelling media

In order to assess which type of medium supported the most efficient labelling of cells with ^{51}chromium, 1x10^{7} RK13 cells were labelled with 200µCi (units of radioactive activity used throughout this thesis will be Curies) ^{51}Cr in cRPMI, in undiluted FBS or in serum-free HBSS. The labelling efficiencies (i.e. the counts per minute per cell) of each set of cells were calculated (Table 2.1).

Labelling medium	cpm per cell
cRPMI	0.095
FBS	0.121
HBSS	0.079

Table 2.1: The labelling efficiencies of RK13 cells labelled with ^{51}Chromium using different media.

After labelling, each set of cells was plated at 1x10^{5} cells per well and evaluated for spontaneous and total releasable ^{51}Cr after 0, 2 and 4 hours incubation at 37°C (i.e. under CTL assay conditions).

	% Spontaneous release Time after labelling (hrs)		
Labelling medium	**0**	**2**	**4**
cRPMI	20.27	27.23	21.99
FBS	22.89	29.22	35.67
HBSS	24.49	ND	51.22

Table 2.2: Spontaneous release of ^{51}Chromium from labelled RK13 cells.

It is clear from the results in tables 2.1 and 2.2 that, although the cells labelled in cRPMI did not label as efficiently as those in FBS, they did not leak as much chromium over the period of a typical CTL assay. As the spontaneous leakage of label is a critical parameter in this type of assay, cRPMI was used for all subsequent labelling procedures.

In another experiment designed to derive optimum conditions for labelling of cells with ^{51}Cr, equine PBMC were labelled and washed as above. They were then incubated in 25ml of cRPMI for various times, washed and then evaluated for spontaneous and total release of label after 4 hours in culture.

	% Spontaneous release of ^{51}Cr	
Bulk culture time	**0 hours**	**4 hours**
0 hours	17.3%	19.5%
1 hours	7.7%	11.7%
2 hours	9.5%	16.0%

Table 2.3. % Spontaneous release for label of PBMC incubated in bulk for various times after labelling.

The results in table 2.3 show that a bulk incubation of labelled target cells for 1 hour in cRPMI followed by washing, markedly reduces the percent spontaneous release of label. Consequently, all short term labelling procedures included this extra incubation period.

2.3.2. Equine peritoneal macrophages as CTL targets

PM from young adult mice have been used as targets in the measurement of CTL against lymphocytic choriomeningitis virus and ectromelia virus (Zinkernagel & Doherty, 1975). Equine peritoneal fluid is relatively easy to obtain and the PM contained in the fluid are readily isolated. However, unlike peritoneal fluid from mice, equine fluid contains up to 50% polymorphonuclear neutrophils (PMN) (Brownlow *et. al.*, 1981. Brownlow, 1982 & 1983). Between 10ml and 20ml of peritoneal fluid containing between $2x10^7$ and $2x10^8$ cells was obtained from each animal. Typically, when PM were isolated using adherence to polystyrene the

recovery of macrophages, identified by morphology, was around 30% of the starting population. Isolation of macrophages using Ficoll hypaque, which depletes PMN, yielded around 10% of the starting population (data not shown).

Productive infection of isolated equine PM with EHV-1 strain Ab4 was demonstrated by infecting PM at an moi of 2.5, for 1 hour and collecting cell-free supernatants at time periods post-infection. The cell-free supernatants were then titrated using RK13 indicator cells.

	$TCID_{50}ml^{-1}$	
	Infected	**Mock inf**
Inoculum	6.35	0
0 hours	1.85	0
22 hours	3.85	0
29 hours	4.85	0
48 hours	4.35	0

Table 2.4. Release of live EHV-1 strain Ab4 from infected PM.

EHV-1 antigen expression was demonstrated on the cell surface of infected PM using the antibody binding assay described in 2.2.5. (data not shown). However, this assay did not allow the assessment of the percentage of infected cells in the PM population. Immunoperoxidase staining of cytospins for EHV-1 antigen, using a rabbit polyclonal

antiserum, showed that approximately 15% of the PM were infected (Fig 2.1).

Infected and mock-infected PM were labelled with ^{51}Cr and the spontaneous and total release values were calculated after 0, 3 and 6 hours using $5x10^4$ cells $well^{-1}$.

	Infected	**Mock Infected**
Time	**Spon Tot %S**	**Spon Tot %S**
0 hrs	387 2,789 13.9	688 4,984 13.8
3 hrs	1,344 3,027 21.2	1,154 5,457 21.2
6 hrs	785 2,988 26.3	1,456 5,349 27.2

Table 2.5. Spontaneous release of ^{51}Cr from PM.
Spon = Spontaneous release of ^{51}Cr. (cpm)
Tot = Total releasable ^{51}Cr.(cpm).
%S = % Spontaneous release of ^{51}Cr (Spon/Tot x 100).

It is clear from the data presented above that PM were infectable with EHV-1 and they did not release large amounts of incorporated label over the period of a typical CTL assay. However, the problems of the low yield of cells, the low percentage of cells infectable with EHV-1 and the need for peritoneal lavage mean that PM were of little use as CTL target cells.

2.3.3. Vascular Endothelial cells as CTL targets

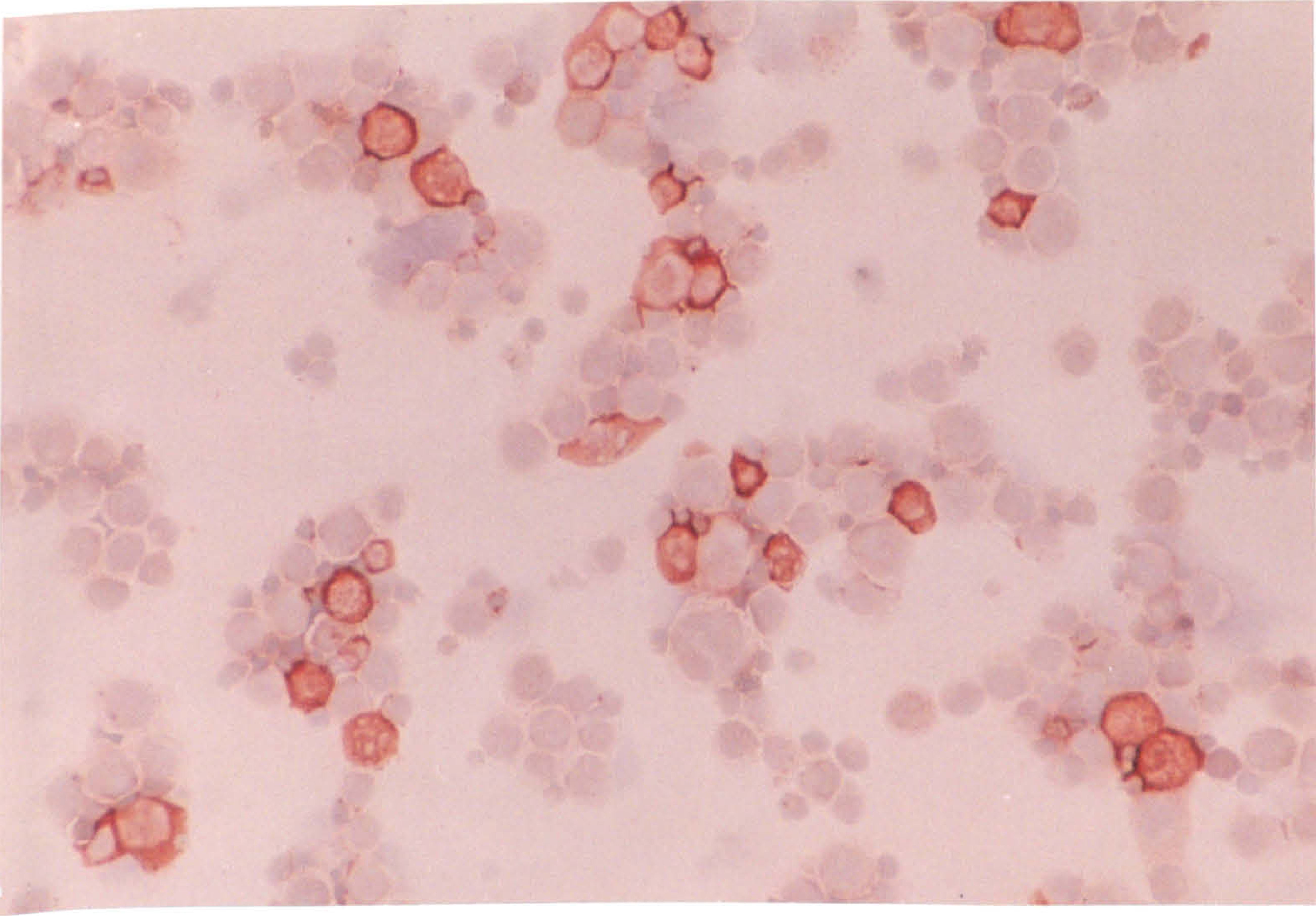
The tropism of EHV-1 (AB4) for endothelial cells *in vivo* (Edington *et. al.*, 1986)

Figure 2.1. Immunoperoxidase staining of EHV-1 strain Ab4 infected peritoneal cells.

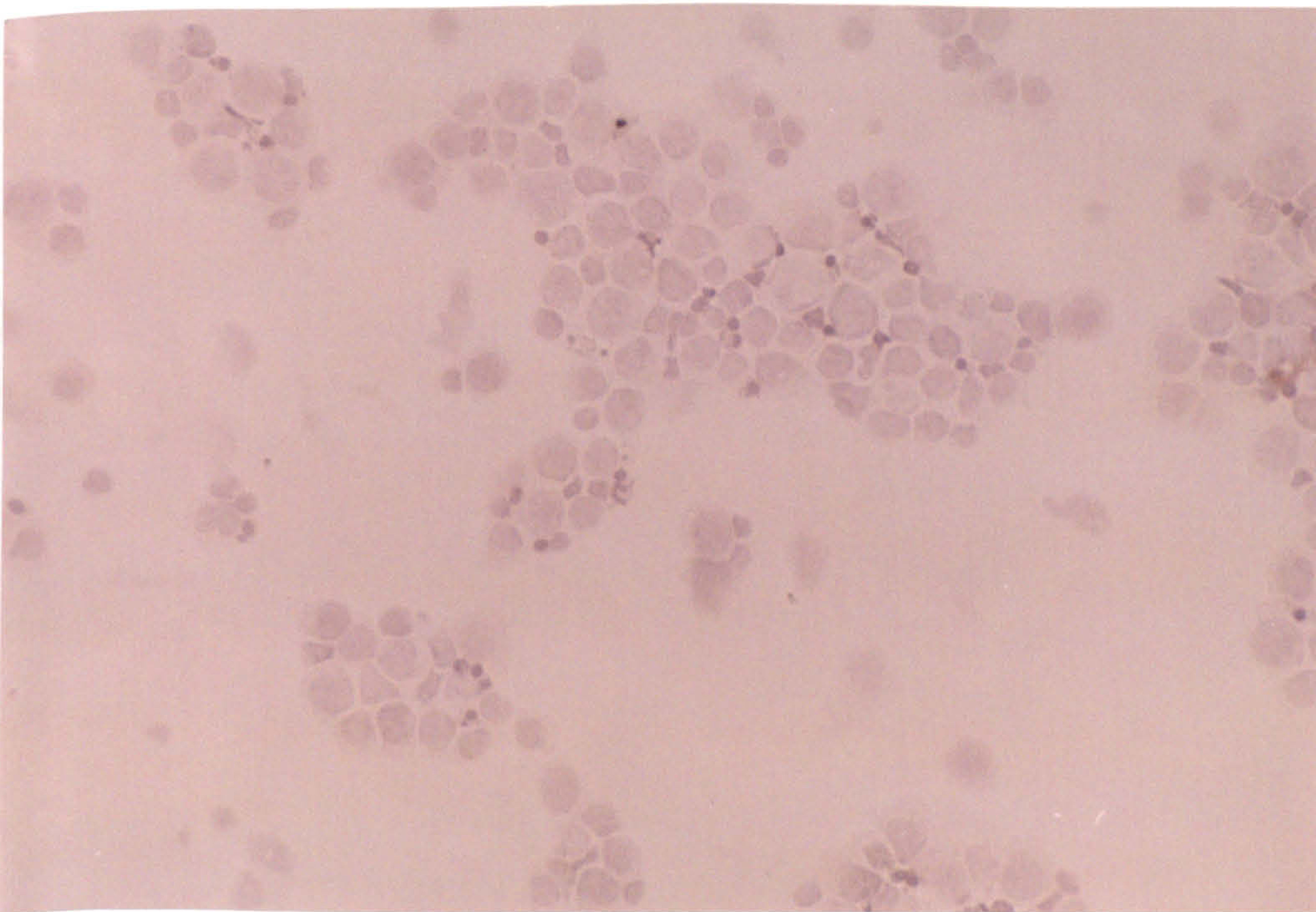
a) Rabbit anti EHV-1 strain Ab4.

b) Normal Rabbit immunoglobulins.

a)



b)



suggests that this cell type would be a relevant target for CTL *in vitro*. Vascular endothelial cell (VEC) lines were derived from the blood vessels of a horse obtained immediately following slaughter. The cell lines were identified as endothelial cells by their expression of cell surface von Willibrands factor (data not shown). VEC were adherent and were therefore easy to grow as a monolayer culture.

VEC were readily infected with EHV-1 strain Ab4. Cytopathic effect was visible by 24 hours and widespread after about 36 hours after infection of the cell sheet. This infection was productive as shown by a 100-fold increase in released virus from washed, infected cells 24 hours after infection (data not shown). Cell surface antigen expression was demonstrated using a mouse monoclonal antibody specific for glycoprotein C (gC, Sinclair, 1991) of EHV-1 in the antibody binding assay. Cells were plated at $2x10^5$ viable cells $well^{-1}$.

	Counts per minute (+/- %CV)	
Antibody	**Infected**	**Mock infected**
Rabbit anti EHV-1	6,188 +/- 9.35%	983 +/- 4.41%
Monoclonal anti gC	11,169 +/- 3.13%	811 +/- 20.3%

Table 2.6. Expression of viral antigens on the surface of VEC.
%CV = Percent coefficient of variation.

Infected and mock-infected VEC ($5x10^4$ cells $well^{-1}$) were labelled with ^{51}Cr and the

spontaneous and total release values were calculated after 0, 3 and 6 hours. Because of the slow uptake of label by these cell it was necessary to label VEC with ^{51}Cr for 18 hours in order to incorporate sufficient label.

	Infected			**Mock-Infected**		
Time	**Spon**	**Tot**	**%S**	**Spon**	**Tot**	**%S**
0 hrs	484	3,802	12.7	725	4,724	15.3
3 hrs	1,442	3,979	36.2	1,970	4,983	39.5
6 hrs	1,828	4,074	44.9	2,585	5,204	49.7

Table 2.7. Spontaneous release of ^{51}Cr from VEC.
The abbreviations are as in Table 4.

Although these cells were directly relevant to EHV-1 infection *in vivo*, they were not of practical use as CTL targets because they released over 35% of the incorporated label within the time period of the assay. Also, it was not possible to isolate VEC from living horses.

2.3.4. *In vitro* matured PBMC (blast-like cells) as CTL targets

Equine PBMC cultured *in vitro* in medium containing 20% autologous serum with no mitogenic or antigenic stimulation for up to 1 month showed the development of large blast-like cells. The majority of these large cells expressed a macrophage phenotype as demonstrated in cytospin preparations using the monoclonal antibody COR CZ3.3

which according to the First International Workshop on equine leukocyte antigens (Kydd & Antczak, 1991. Kydd *et. al.*, 1994c) is specific for a marker present on macrophages.

Infection of these cells was demonstrated in cytospin preparations using the immunoperoxidase method outlined above. The percentage of the cells expressing viral antigen after 20 hours of infection was approximately 15% (Fig 2.2).

In vitro matured PBMC were derived from three animals, infected with EHV-1 strain Ab4 at an moi of 10 and labelled with ^{51}Cr for 90 min. Spontaneous and total release values were calculated after 6 hours incubation of $5x10^4$ cells $well^{-1}$.

	Infected			Mock Infected		
Horse #	**Spon**	**Tot**	**%S**	**Spon**	**Tot**	**%S**
# 223	334	1,199	27.9	369	1,884	19.6
# 370	854	3,962	21.6	950	8,092	11.7
# 382	722	4,884	14.8	911	8,197	11.1

Table 2.8. Spontaneous release of ^{51}Cr from *in vitro* matured PBMC.
The abbreviations are as in table 4.

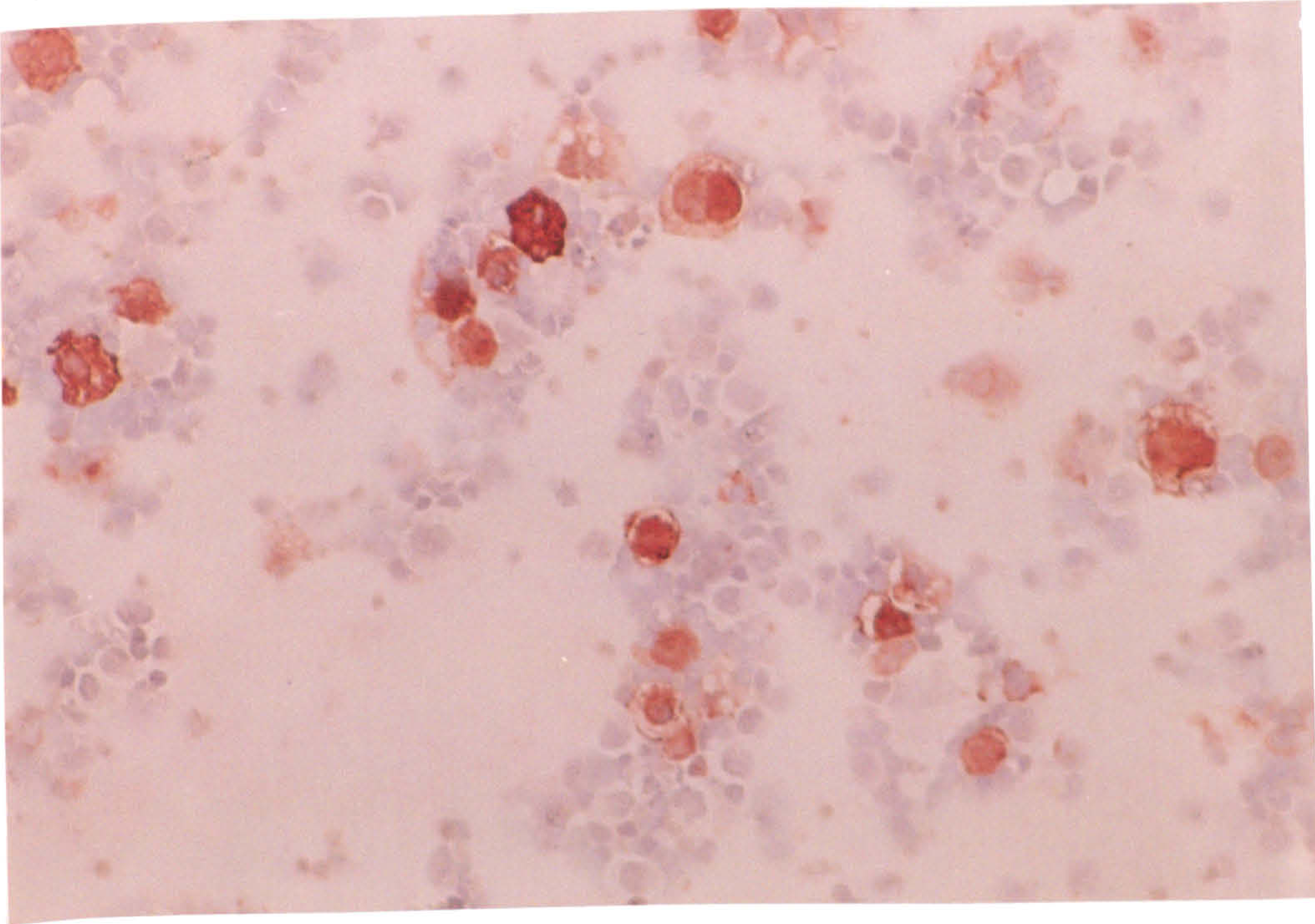
The % spontaneous release values were within acceptable ranges. The labelling efficiencies of the macrophage-like cells from the three animals used varied

Figure 2.2. Immunoperoxidase staining of EHV-1 strain Ab4 infected *in vitro* matured PBMC.

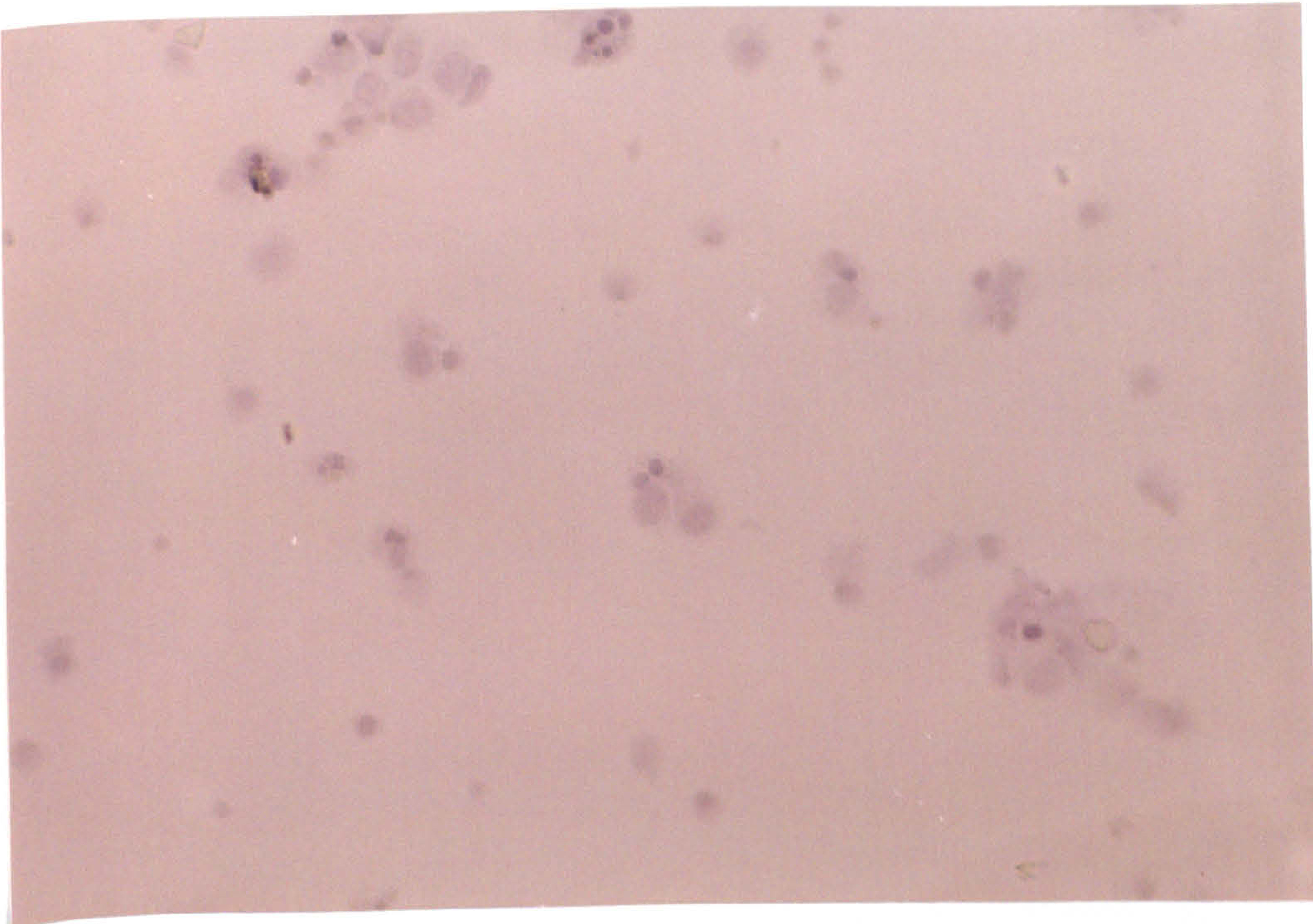
a) Rabbit anti EHV-1 strain Ab4.

b) Normal Rabbit immunoglobulins.

a)



b)



considerably ranging from 0.012 counts per cell (pony #223 infected cells) to 0.109 counts per cell (pony #370 mock-infected cells). This suggested that the populations of these cells derived from different animals were heterogeneous. This was confirmed using FACS analysis of *in vitro* matured cells derived from a range of animals (table 2.9). Unfortunately, antibody COR CZ3.3 did not function in FACS analysis (J. Kydd, AHT, Newmarket, personal communication), therefore the heterogeneity of cell populations was confirmed using T and B cell markers.

	Monoclonal antibody specificity			
Pony	**CD2**	**CD4**	**CD8**	**B cell**
165	17.8%	14.2%	2.7%	0.0%
168	6.5%	4.4%	1.9%	0.2%
232	10.2%	8.4%	3.0%	0.3%
236	7.4%	3.9%	2.6%	0.0%
259	22.1%	12.2%	4.0%	0.2%
270	15.1%	5.4%	5.2%	0.1%
276	20.4%	16.3%	3.6%	0.0%
PBMC	76.4%	53.3%	19.5%	21.1%

Table 2.9. Heterogeneity of *in vitro* matured macrophage-like cells.
The FACS gates were set on the large lymphocyte population. The results refer to the percentage of cells which stained positive with each monoclonal antibody.

The results in Table 2.9 showed that cells matured *in vitro* developed into populations which were phenotypically different from fresh PBMC preparations. Matured cells from the seven ponies tested contained between 6.5% and 22.1% T cells ($EqCD2^{+}$ cells) and very few B cells. This implies that the remainder of the cells in the matured populations were of non-T, non-B cell origin. The cells matured from PBMC from different animals did not develop into populations of consistent composition despite being cultured under identical conditions. If these cells were to be used in a CTL assay any differences in the % specific cytotoxicity between animals could be attributed to the heterogeneity of the target cells rather than to differences in the CTL activity. Because of this and the relatively low percentage of cells expressing viral antigens after infection (20-30%) these macrophage-like cells would not make good CTL targets.

2.3.5. Equine blast cells as CTL targets

Equine PBMC were assessed for their ability to become infected with EHV-1. Immunoperoxidase staining of infected PBMC (moi of 10 and cultured for 18 hours) with a rabbit anti EHV-1 antiserum (extensively pre-absorbed against uninfected PBMC) showed that between 10% and 20% of the total cells expressed viral antigens. On stimulation of PBMC with $2.5\mu g ml^{-1}$ PHA this increased slightly to about 30% of the total cells. Dr. G. Allen (University of Kentucky) suggested that the low percentage of infected cells in the blast population was probably due to their reversion back to the resting state during the 18 hour infection period after the stimulus had

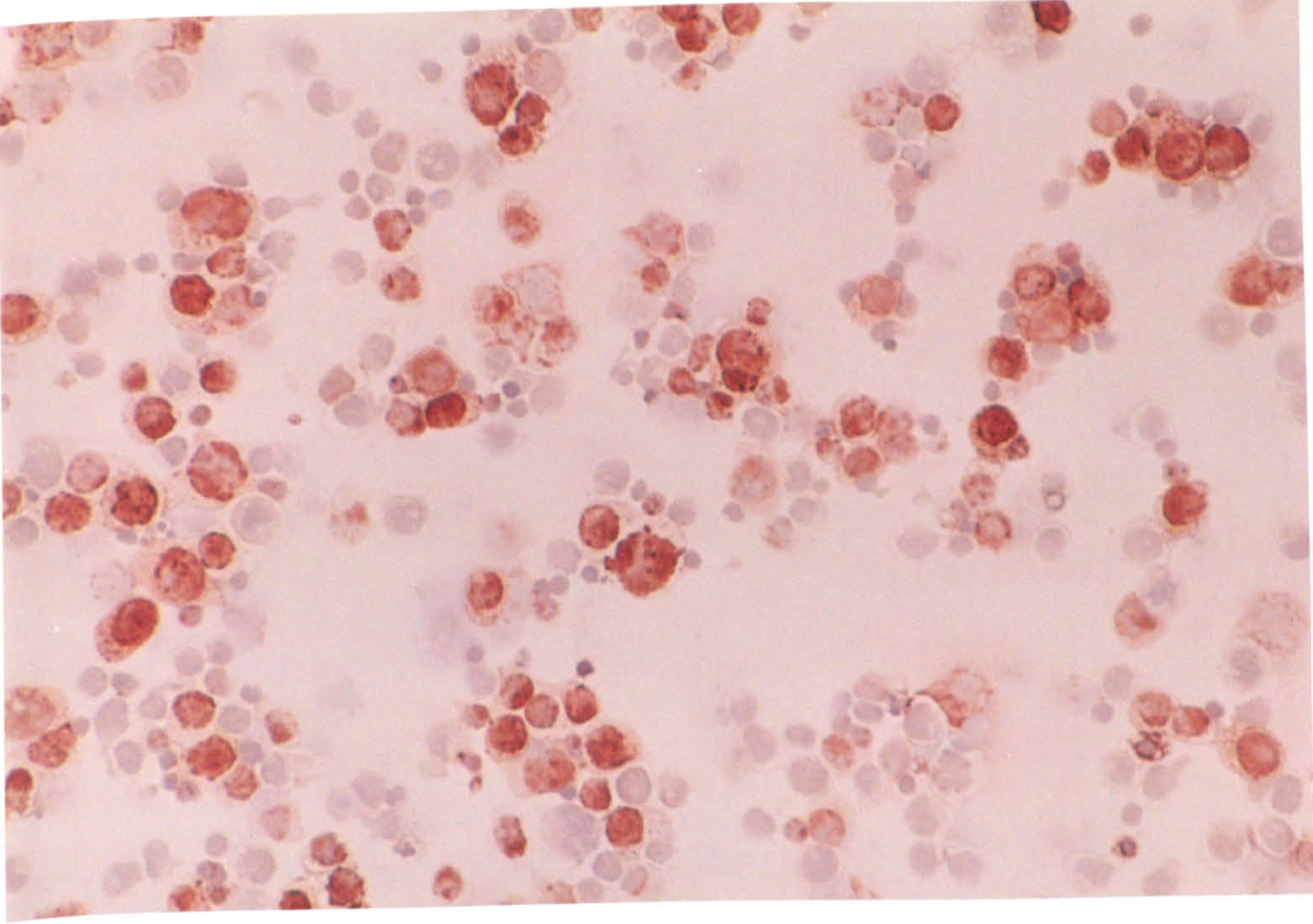
been removed. Following Dr. Allen's advice, 48 hour blast cells were produced using $2.5\mu g ml^{-1}$ PWM in AIM-V/SM medium (Gibco). These cells were then infected at a multiplicity of infection of 10 for 14 hours in the presence of 20 international units ml^{-1} of recombinant human interleukin 2 (rhIL2). After infection cytospins were produced and stained with the absorbed rabbit anti EHV-1 antiserum. As can be seen in Fig 2.3, a large proportion of the cells in the blast cell population expressed viral antigen on the cell surface. This was also the case when other mitogens (e.g. PHA) were used (Fig 2.4). Again, on the advice of Dr. Allen, these cells were labelled with ^{51}Cr during 12 hours of the infection period. Aliquots of $2.5x10^6$ cells ml^{-1} were infected for 1.5 hours with EHV-1 at an moi of 10, washed and labelled with $150\mu Ci$ of ^{51}Cr. The cells were incubated with the isotope for 12 hours and evaluated for spontaneous and total release of label over a 4 hour period. Cells were tested at $1x10^4$ cells $well^{-1}$.

Figure 2.3. Immunoperoxidase staining of EHV-1 strain Ab4 infected PWM blast cells.

a) Rabbit anti EHV-1 strain Ab4.

b) Normal Rabbit immunoglobulins.

a)



b)

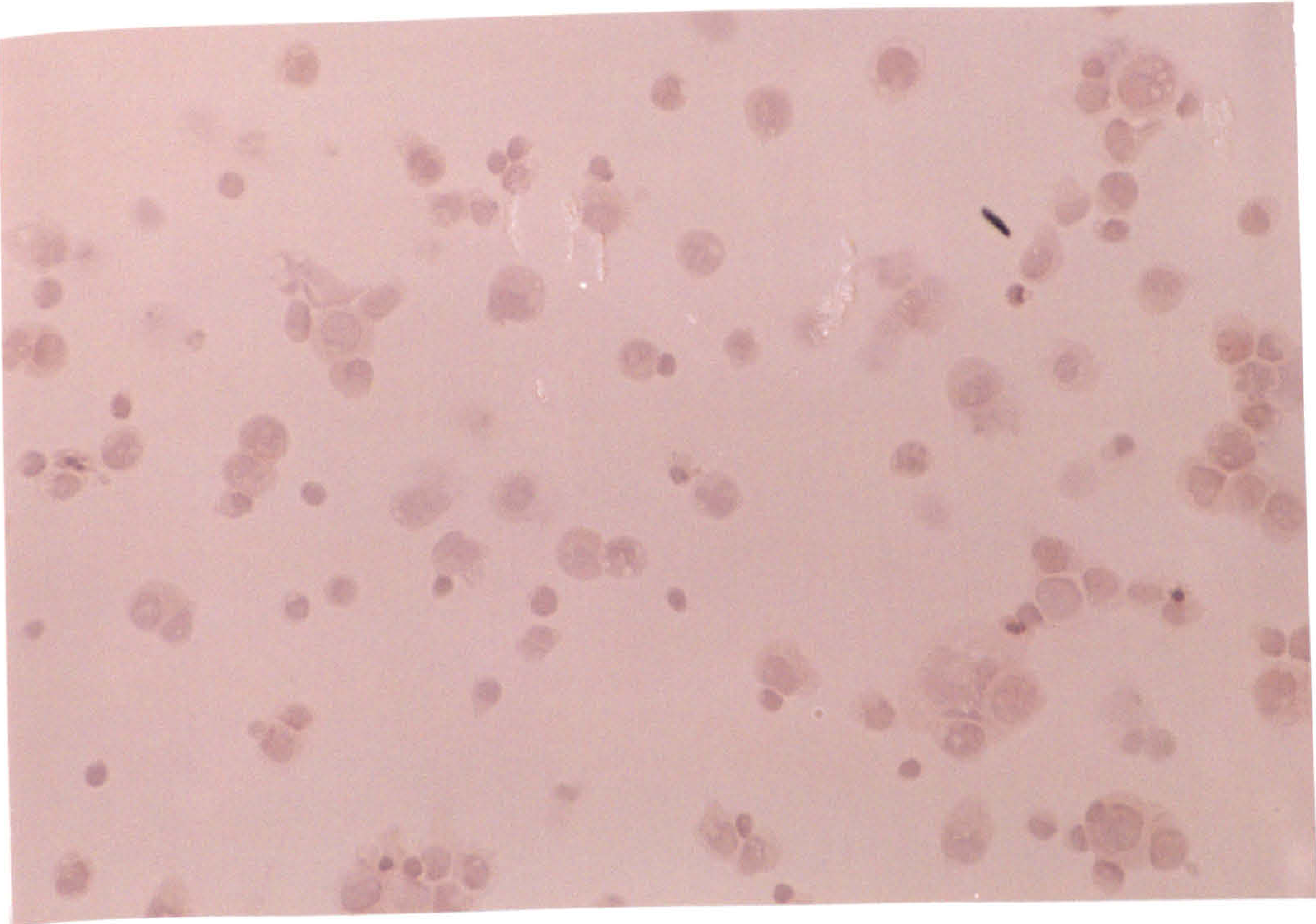
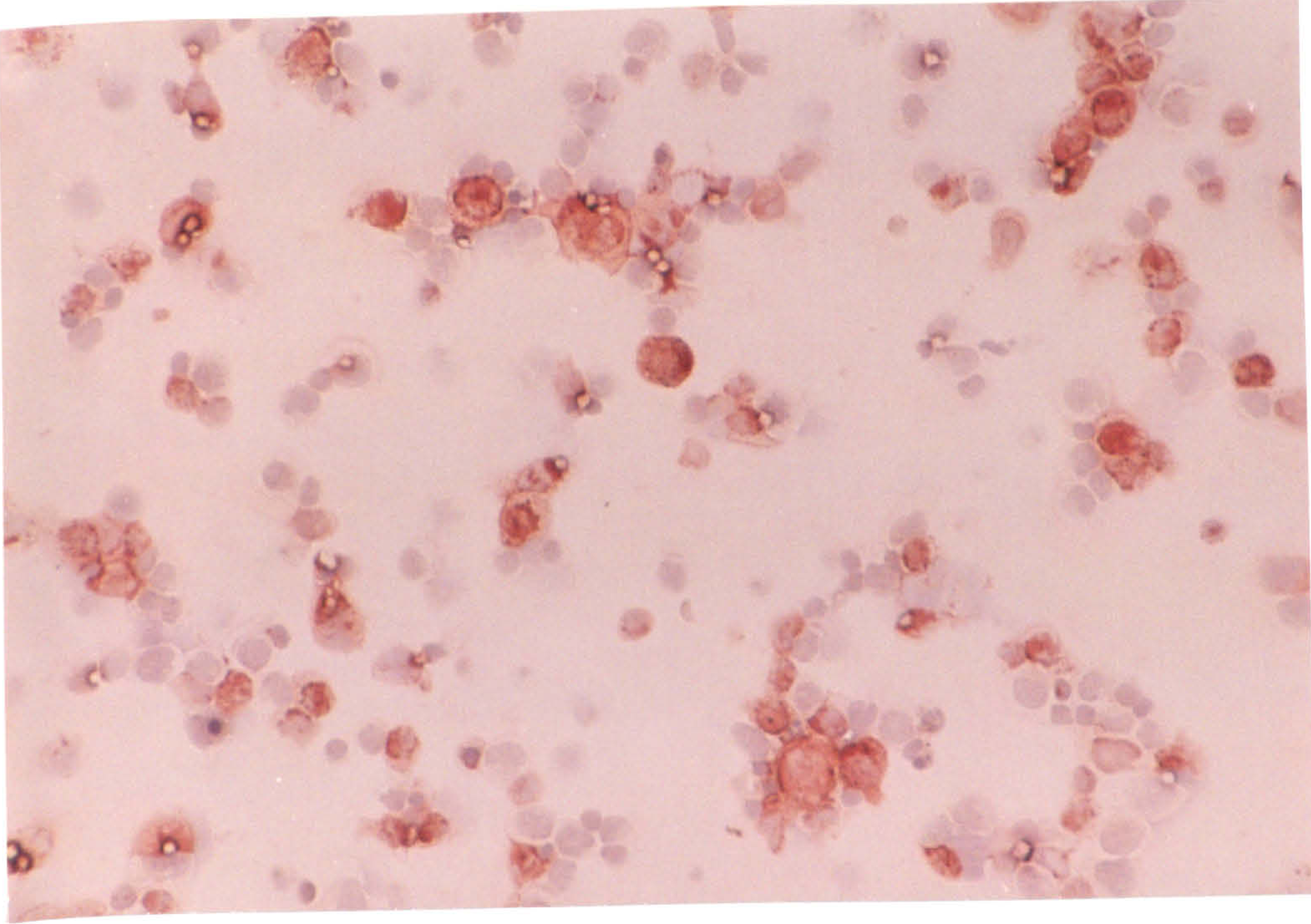


Figure 2.4. Immunoperoxidase staining of EHV-1 strain Ab4 infected PHA blast cells.

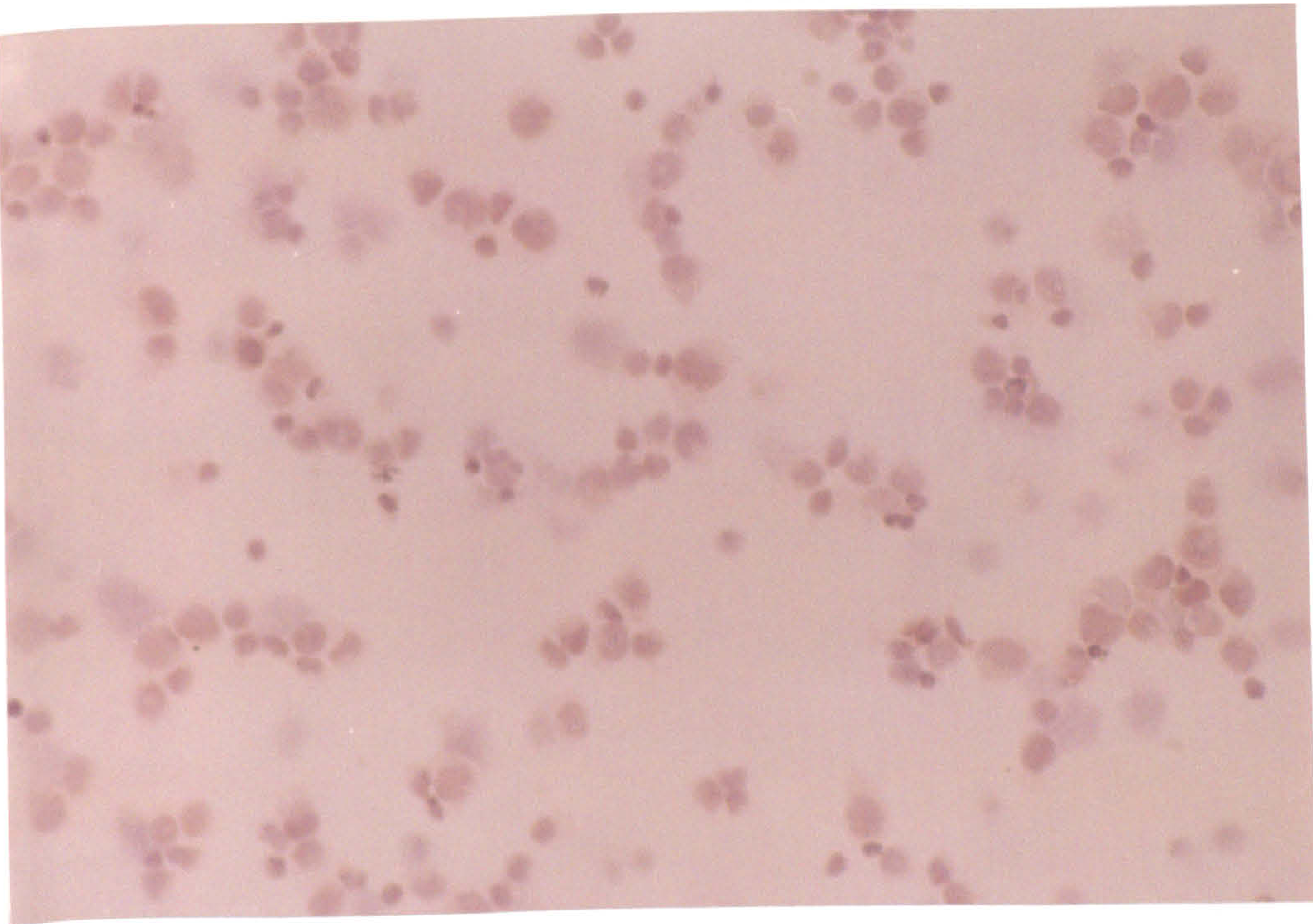
a) Rabbit anti EHV-1 strain Ab4.

b) Normal Rabbit immunoglobulins.

a)



b)



	Infected			Mock-Infected		
Horse	**Spon**	**Tot**	**%S**	**Spon**	**Tot**	**%S**
219	301	1,535	19.6	256	1,032	24.8
302	249	1,149	21.7	454	1,522	29.8
168f	163	1,249	13.0	144	1,071	13.4
701	113	760	14.9	112	855	13.1
C1	206	1,040	19.8	206	1,227	16.8
235	195	1,012	19.2	173	694	24.9

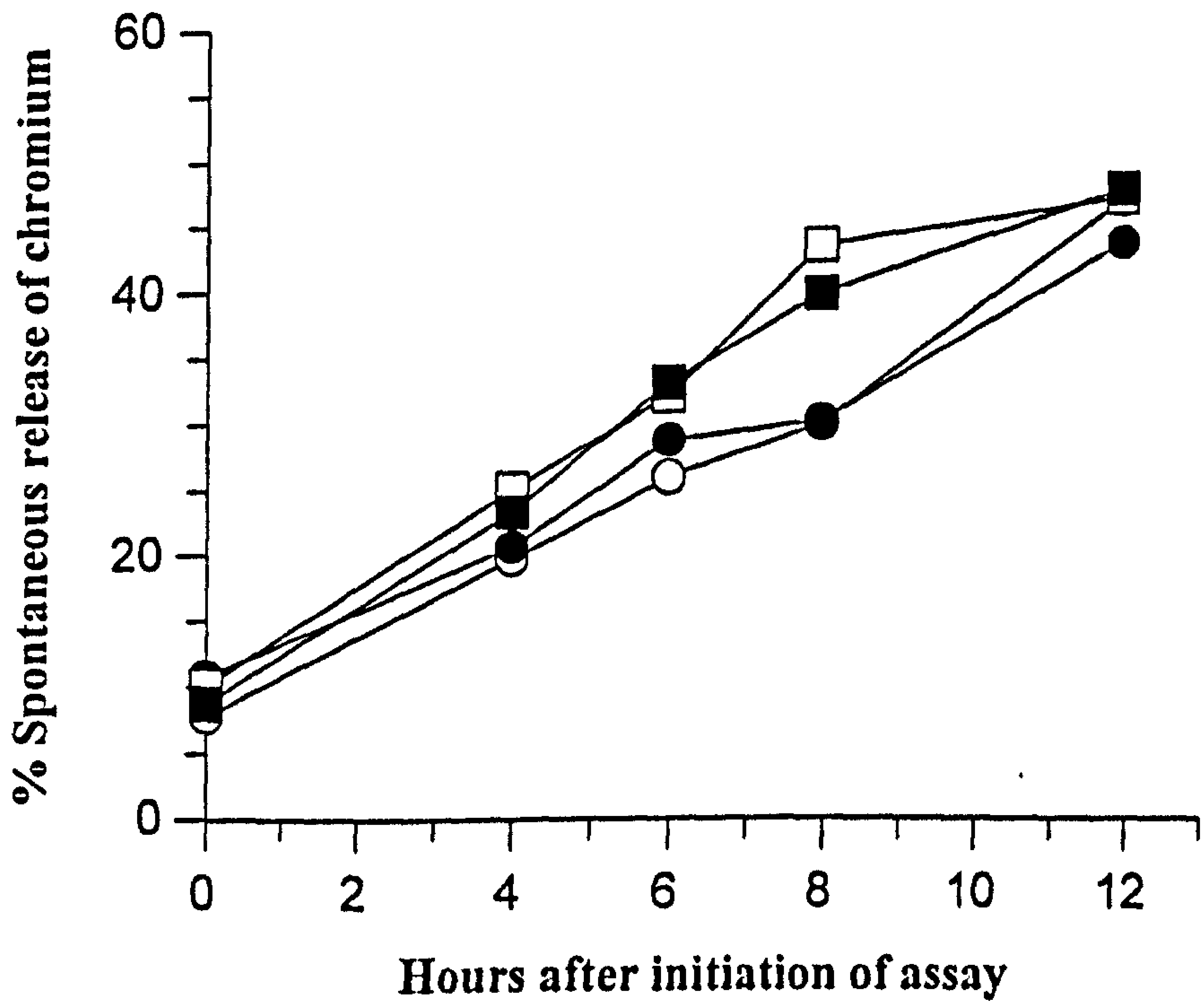
Table 2.10. Spontaneous release of ^{51}Cr from PWM blast cells after 4 hours. The abbreviations are as in table 4.

The spontaneous release values shown in Table 2.10 were within acceptable limits for use in CTL assays. The spontaneous release characteristics were further studied over a longer period of time in order to determine the time limit for a CTL assay using these cells as targets as shown in Fig 2.5. PWM blast cells released over 30% of label when they were incubated for longer than about 6 hours. However, as the majority of CTL assays are between 4 and 6 hours in duration, this was not thought to be a problem.

An initial assay of CTL activity in an immune animal was carried out using the induction culture method developed by Dr. Allen (Allen *et. al.*, 1995). The results of

Figure 2.5. Spontaneous release of radioactive chromium from labelled equine PWM blast cells.

open circles = mare #1 infected blast cells.
closed circles = mare #1 mock-infected blast cells.
open squares = mare #2 infected blast cells.
closed squares = mare #2 mock-infected blast cells.


% Spontaneous release of chromium
60
40
20
0
0
2
4
6
8
10
12
Hours after initiation of assay

this assay can be seen in Figures 2.6 and 2.7. Equine PWM blast cells were susceptible to lysis by EHV-1 induced CTL effector cells (mare #302 in Fig 2.6a). Effector cells from an MHC class I mis-matched horse (mare #219 in Fig 2.6a) did not kill infected targets, although they did kill autologous (*i.e.* mare #219), infected targets (Fig. 2.7). Autologous mock-infected target cells were not susceptible to killing by EHV-1 induced CTL (Figs. 2.6b and 2.7b).

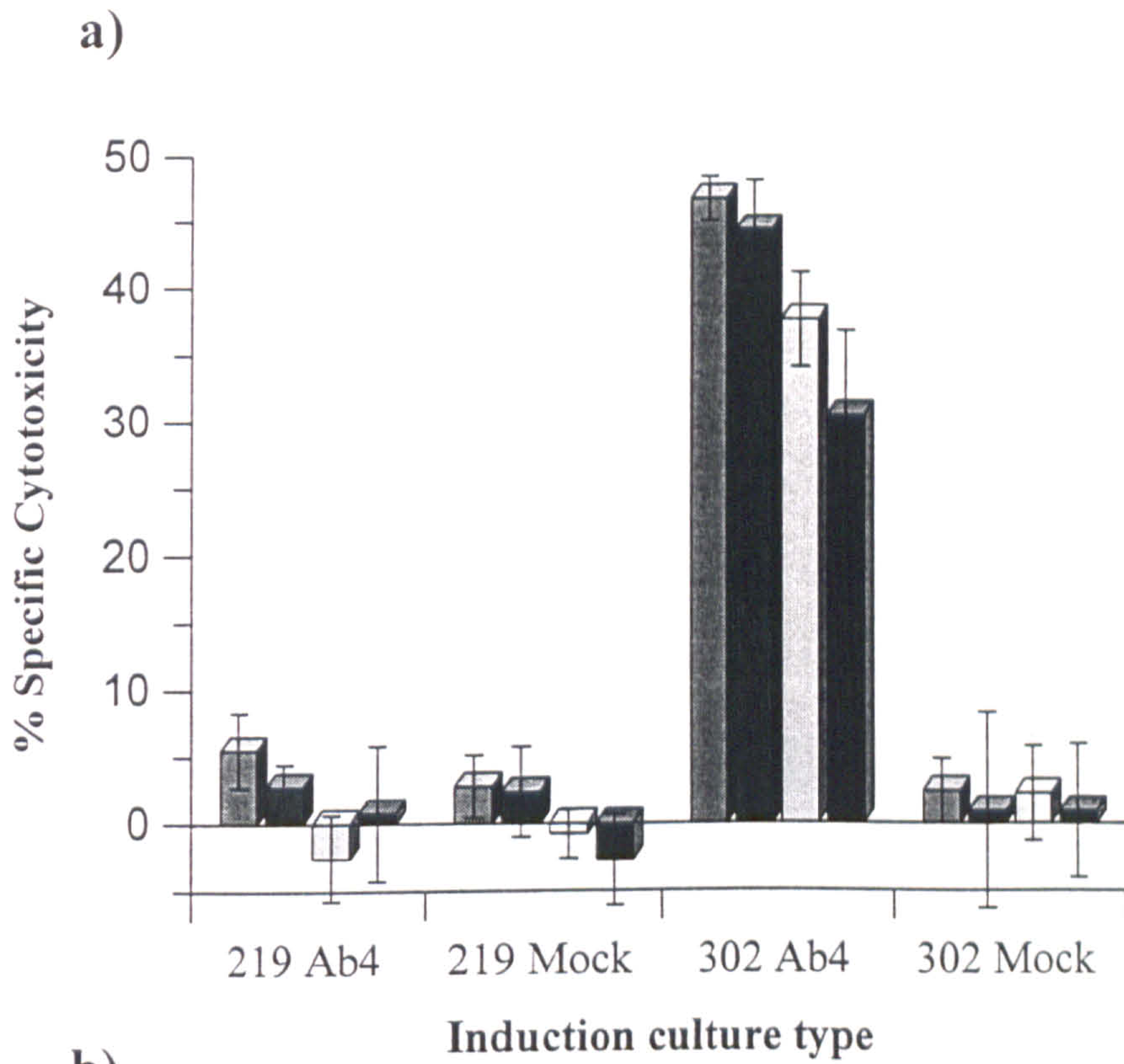
2.4. Discussion

A wide range of target cells have been used by researchers to quantify CTL activity. The study of CTL in inbred mouse models of disease has spawned the production of many MHC typed cell lines. For example, Gao *et. al.*, (1991) used the murine tumour cell lines P815 (H-2^d), EL4 (H-2^b), and BW5147 (H-2^k) to demonstrate CTL induced by priming mice with short synthetic peptides of influenza virus. These target cells were easily grown *in vitro* in tissue culture. The wide availability of tissue typing reagents has meant that target cells for use in the study of human CTL can easily be matched to a particular patient's MHC type and grown in tissue culture for use in CTL assays. The use of Epstein Barr virus (EBV) to transform human PBMC into continuously growing B cell lymphoblastoid cell lines (B-LCL) has also improved the capacity to measure human CTL specific for a number of viruses (Cherrie *et. al.*, 1992, Posavad and Rosenthal, 1992). The study of virus-specific CTL in veterinary species has been compromised due to the lack of suitable target cells. Hence, cell lines suitable as target cells for the study of viruses important in sheep, cattle or horses are not available. Also, MHC typing of cells from these species is not always

Figure 2.6. EHV-1 specific CTL from mare #302 assayed using PWM blast cells as targets.

a) Autologous, EHV-1 infected targets.

b) Autologous, mock-infected targets.


a)
50
40
30
20
10
0
% Specific Cytotoxicity
219 Ab4
219 Mock
302 Ab4
302 Mock
Induction culture type

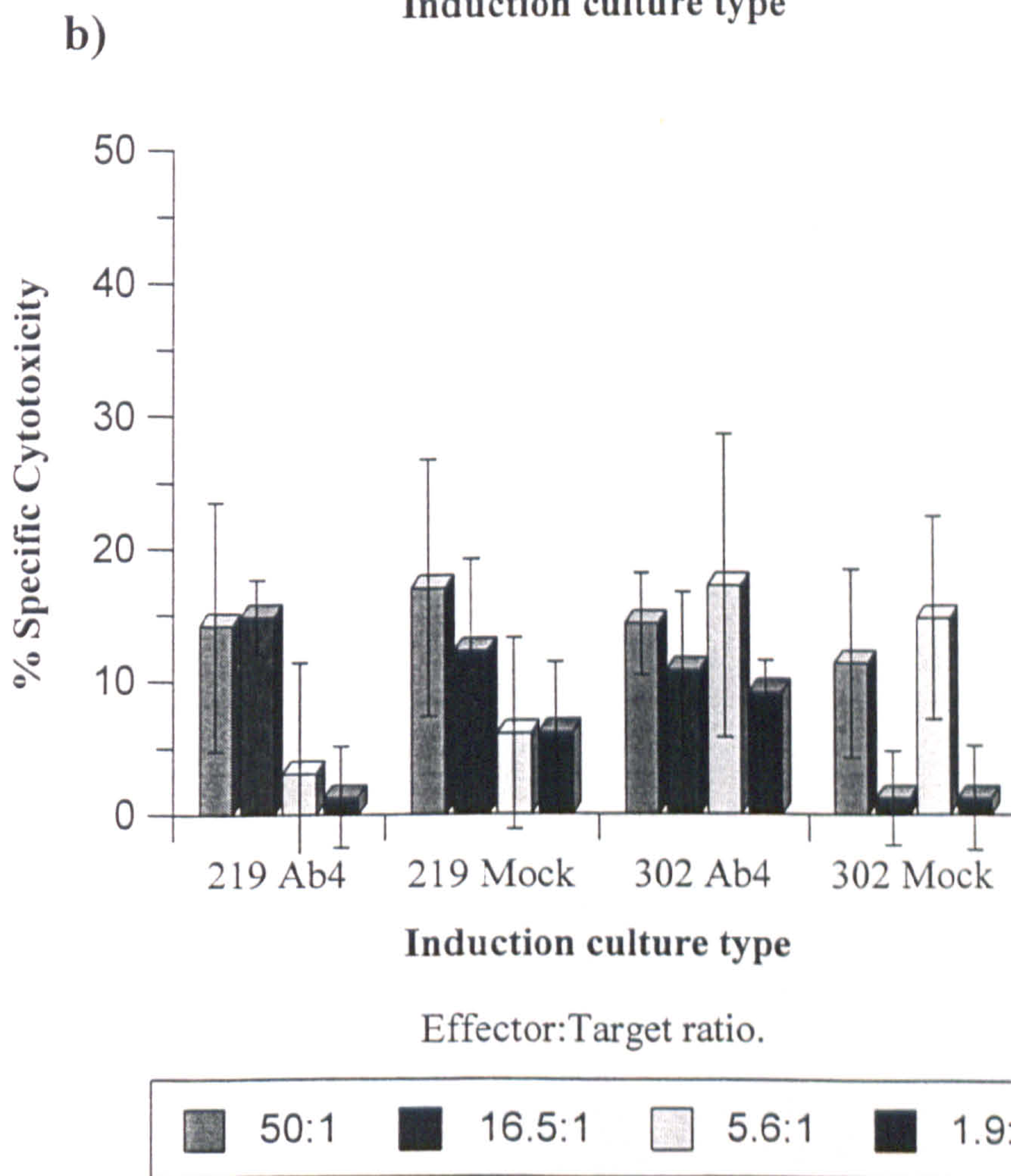
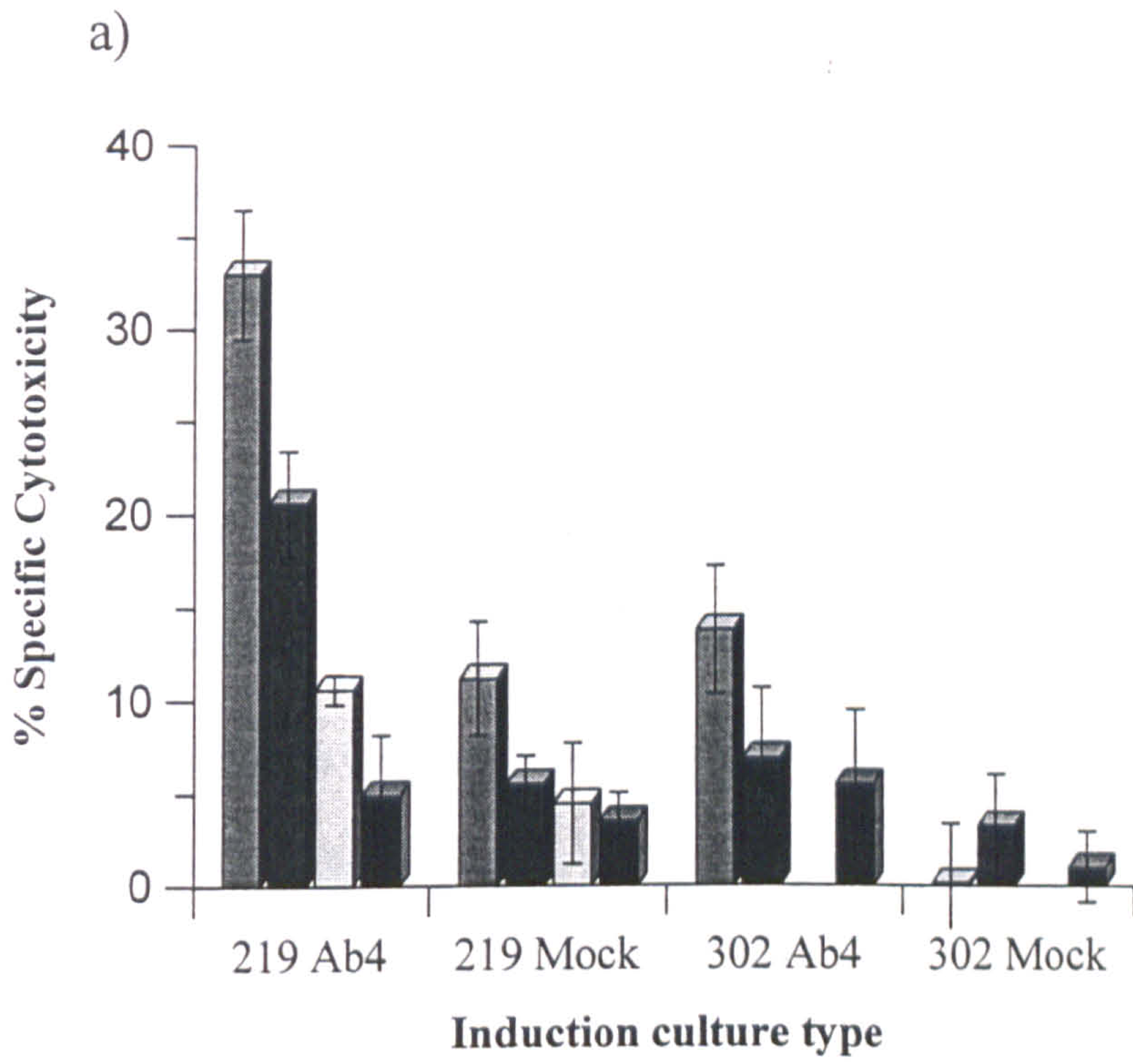

b)
50
40
30
20
10
0
% Specific Cytotoxicity
219 Ab4
219 Mock
302 Ab4
302 Mock
Induction culture type
Effector:Target ratio.
50:1
16.5:1
5.6:1
1.9:1

Figure 2.7. EHV-1 specific CTL from mare #219 assayed using PWM blast cells as targets.

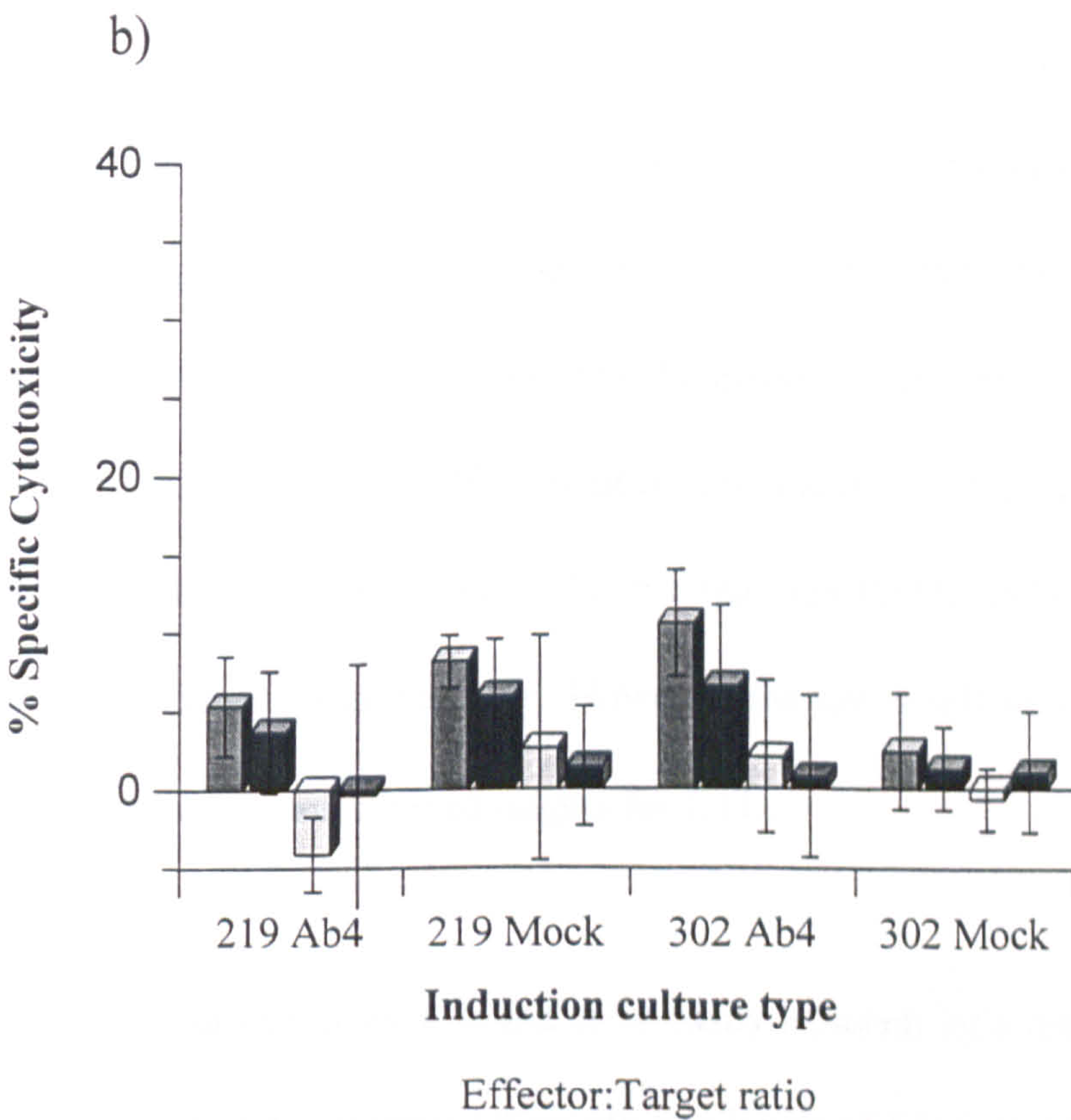
a) Autologous, EHV-1 infected targets.

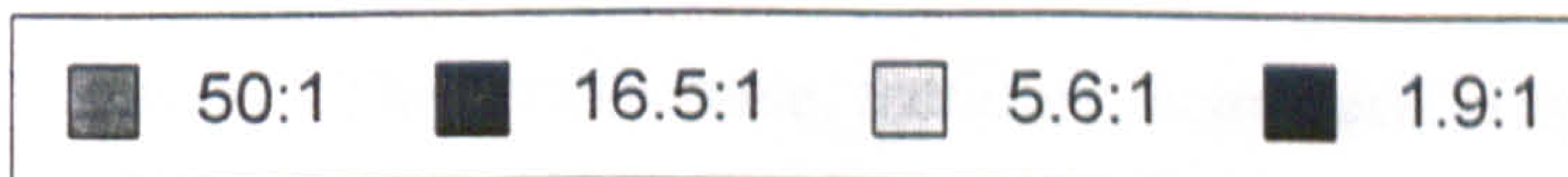
b) Autologous, mock-infected targets.

a)



b)





possible. Sharma and Woldehiwet (1991) studied bovine respiratory syncytial virus specific CTL derived from sheep using cultured lamb testis cells as targets, whilst McGuire *et. al.*, (1994) studied equine infectious anaemia virus specific CTL using autologous primary equine kidney cells.

It is clear from the results presented in this chapter, that the most promising cell type for use in an EHV-1 specific CTL assay is the PWM-stimulated blast cell. The use of PWM is not a prerequisite as other mitogens, *e.g.* PHA and Con A, can be used. Mitogen-stimulated blast cells are easy to produce, support replication of EHV-1 in a high proportion of the cells and retain ^{51}Cr long enough for an assay to be completed.

Of the other cell types tested, PM labelled well and did not release significant amounts of ^{51}Cr spontaneously. However, their low levels of infection and the low yield of viable macrophages from whole peritoneal fluid meant that they were unsuitable for large studies of EHV-1 specific CTL activity. VEC proved to be of little use as CTL targets due to the difficulty of isolation and their high spontaneous release of label (about 35% after 3 hours). *In vitro* matured PBMC did not release excessive amounts of label spontaneously. However, the low levels of infection of these cells rendered them unsuitable as targets for CTL.

In conclusion, PWM blast cells were found to be easily isolated, infected and labelled with ^{51}Cr. Furthermore, they released relatively low levels of label spontaneously over a four hour CTL assay. These cells have, therefore, been used to optimise an

EHV-1 specific CTL assay, to determine the phenotype of the cells responsible for CTL mediated killing in this system, to screen a large number of horses with diverse infection histories in order to determine their immune status in terms of CTL activity and to develop CTL LDA methods in order to determine the frequencies of CTL precursors present before and after infection with EHV-1 and EHV-4.

3. Development of the CTL assay

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3.1. Introduction

The ^{51}Chromium (^{51}Cr) release assay was first applied to the study of the cellular mechanisms involved in immunity when Brunner *et. al.* (1968) used it to measure the *in vitro* cytotoxic effect of spleen cells on ^{51}Cr labelled, allogeneic target cells. Since then the assay has been used extensively to study the cytolytic activity of a wide range of cell types.

One of the most widely used applications for the ^{51}Cr release assay is in the study of cytotoxic lymphocytes which are involved in protection against and recovery from infectious diseases. As outlined in chapter 1, these lymphocytes are functionally and phenotypically diverse but fall into two main types, the natural killer (NK) cell and the cytotoxic T lymphocyte (CTL). The use of the assay to study virus-specific CTL began with the work of Zinkernagel and Doherty when they demonstrated that the T cell-mediated cytolytic response to lymphocytic choriomeningitis virus (LCMV) of mice was virus-specific and genetically restricted (Zinkernagel and Doherty, 1974). The role of anti-viral CTL in protection against and recovery from infection was initially studied using LCMV infection of mice as a model. In this model, CTL were generated which were directly measurable *ex vivo* from spleens of infected mice, 6 to 10 days after infection (Marker & Volkert, 1973). Levels of *in vitro* specific lysis of infected target cells mediated by anti LCMV CTL were equivalent to those seen with CTL clones induced with other viruses. There was a distinct immunopathological aspect to LCMV

infection of mice where intracerebral injection with virus caused a lethal chriomeningitis (Doherty & Zinkernagel, 1974). However, T cell deficient mice infected with the virus did not develop choriomeningitis disease but became LCMV carriers. Due to the above properties of LCMV infection of the mouse and in particular to the vigorous CTL response after infection this model has yielded some important findings. Information has been produced concerning the clearance of LCMV *in vivo* using adoptively transferred, cloned anti-viral CTL (Byrne & Oldstone, 1984); the role of polyclonal CTL in the clearance of LCMV from the spleens of infected mice (Lehmann-Grube *et. al.*, 1988); the induction of anti-viral CTL with non-replicating protein antigens derived from LCMV (Bachmann *et. al.*, 1994) and the *in vitro* selection of LCMV escape mutants by CTL (Aebischer *et. al.*, 1991). This work led to the realisation that the biological role of CTL was immune surveillance and elimination of virus infected cells (Doherty and Zinkernagel, 1977) rather than the previously held view that CTL were, simply, effectors of allograft rejection.

The measurement of CTL from the horse has been hindered by the lack of equine reagents. Allospecific CTL were demonstrated after induction in a one way mixed lymphocyte reaction (MLR) culture by O'Brien (PhD thesis, 1992). This established that "classical" CTL activity was measurable in equine leucocytes. Recently, McGuire et. al. have demonstrated equine $CD8^+$ CTL specific for equine infectious anaemia virus (EIAV) in infected horses (McGuire *et. al.*, 1994). The method used in this study was a 17 hour ^{51}Cr release assay using autologous equine kidney cell targets either infected with

EIAV or recombinant vaccinia viruses expressing EIAV genes (env, gag or 5' pol). The effector cells generated in this study did not require any *in vitro* culture in order to kill virus infected target cells. Hannant and Mumford (1989) induced equine influenza virus-specific CTL using an *in vitro* culture which included equine T cell growth factor (TCGF) preparations. The resultant CTL were assayed using autologous and allogeneic, influenza virus-infected PBMC. They found that CTL induced with moderate amounts of TCGF killed both autologous and allogeneic target cells. It was only when the amount of TCGF was reduced to 5% of the induction culture volume that genetically restricted CTL were revealed. The non-genetically restricted CTL measured in this study were *in vitro* induced lymphokine activated killer (LAK) cells. Hormanski *et. al.* (1992), reported that equine LAK cells lysed the equine lymphosarcoma cell line EqT8888, whilst they failed to lyse human (Daudi) or murine (EL4) tumour cell lines. The assay for MHC restricted, EHV-1 specific CTL developed and described in this chapter has avoided the use of TCGF in induction cultures in order to minimise the induction of these types of cells.

EHV-4 specific CTL were described by Bridges & Edington (1987). In this study, equine skin fibroblasts pulsed with inactivated EHV-4 antigen were used as target cells in order to demonstrate genetically restricted CTL. As outlined previously, in order to measure "classical" MHC class I restricted, $CD8^{+}$ CTL, infection of a target cell is absolutely necessary unless the target peptides are available.

This chapter will describe the optimisation and use of a bulk culture assay for the

measurement of EHV-1 specific CTL. The optimised assay used in this study was based on that described by Allen et. al. (1995) who's collaboration is kindly appreciated. In order to maintain clarity of the text, all of the media formulations are described in appendix A.

The aims of the experimental work in this chapter were to develop virus-specific assays for CTL using EHV-1 Ab4 as the *in vitro* inducing antigen and as the infecting virus for target cells. Specific objectives were:

a) to optimise the EHV-1 specific CTL assay in terms of induction culture duration, antigen input and cold target inhibition;

b) to confirm MHC restriction and $CD8^+$ phenotype of EHV-1 specific CTL;

c) to measure CTL activity in a selection of foals maintained in the AHT breeding herd;

d) to measure the development of CTL activity after primary EHV-1 infection of specific pathogen free (SPF) foals;

e) to measure cross-reactive CTL, *i.e.* CTL induced with EHV-4 and assayed using EHV-1 infected target cells;

f) to determine whether immediate early proteins of EHV-1 are recognised by EHV-1 specific CTL.

3.2. Materials and Methods

3.2.1. Isolation of equine PBMC

Equine PBMC were isolated from whole blood using the method described in section 2.2.3. The cells were then resuspended in AIM-V/SM containing 5% heat-inactivated autologous horse serum and counted using a Nueberger haemacytometer. The cells were resuspended at a concentration of $1x10^8$ cells ml^{-1} in AIM-V/SM.

3.2.2. Induction of EHV-1 specific CTL

In order to induce EHV-1 specific CTL *in vitro*, $1.1x10^8$ isolated equine PBMC were cultured in upright T75 tissue culture flasks (Corning) in 30ml of AIM-V/SM containing 5% heat-inactivated autologous horse serum (a concentration of $3.67x10^6$ cells ml^{-1}). Live EHV-1 strain Ab4/9 (1.5ml at a concentration of $1x10^{7.6}$ $TCID_{50}ml^{-1}$) was added to one set of cultures at a concentration approximating to a multiplicity of infection of 0.5. Another set of cultures were mock-induced by substituting mock-infected tissue culture supernatant for the live virus. The cultures were incubated at 37°C for six days in an atmosphere of 5% CO_2 in air. The remaining PBMC at $1x10^8$ cells ml^{-1} were incubated in 5% CO_2 in air for 10 min and kept in tightly closed tubes at 4°C for use as target cells.

3.2.3. EHV-1 specific ^{51}Chromium release assay

On day 3 of the induction culture, PBMC for use as target cells were retrieved from 4°C, washed in HBSS and counted in trypan blue in order to determine the % viability of the

cells. They were then incubated at a concentration of $3x10^6ml^{-1}$ viable cells in 10ml of AIM-V/SM-5% containing $2.5\mu gml^{-1}$ Pokeweed mitogen (PWM, Sigma). These cultures were incubated upright in T25 tissue culture flasks (Corning) for a further 48 hours in order for the PBMC to develop into actively proliferating blast cells.

On day 5 of the induction culture the PWM blast cells were harvested and the dead cells removed by layering them onto a Ficoll Hypaque gradient using the same method as for the isolation of the PBMC except that the resultant band of cells was washed in HBSS containing 20 units ml^{-1} of recombinant human Interleukin 2 (rhIL2) in order to maintain the blast cells in an actively proliferating state. Aliquots ($2.5x10^6$ cells) of the PWM blast cells from autologous and allogeneic sources were either infected with EHV-1 strain Ab4/9 at a multiplicity of infection of greater than 10, or mock-infected, and incubated for 90 min at 37°C in 5% CO_2 in air with frequent gentle resuspension. The target cells were then washed and resuspended in 1.0ml of cRPMI containing 20 units ml^{-1} rhIL2. The cell suspension was transferred to one well of a 48 well culture plate to which $150\mu Ci$ of ^{51}Chromium had been added. The plates were then incubated for 12 hours at 37°C in 5% CO_2 in air.

Approximately 2 hours before the end of the target cell infection period, the induction cultures were harvested and washed twice in HBSS and once in cRPMI. The cells were then counted using trypan blue to determine the viability of the effector cells and resuspended at a concentration of $1x10^7$ viable cells ml^{-1} in cRPMI. The effector cells

were kept on ice. Each effector cell type was titrated in quadruplicate in round bottom microtitre plates from 1×10^6 cells $well^{-1}$ using doubling dilutions. The target cells were harvested and washed twice in HBSS and once in cRPMI. The supernatants were discarded into a radioactive waste bottle after each wash to minimise any radioactive hazard. After the final wash the target cells were resuspended in 2ml of cRPMI and counted in trypan blue to determine the % viability. They were resuspended to give 1×10^5 cell ml^{-1} and 100µl of the cell suspension was counted in a gamma counter to determine the total incorporated ^{51}Cr. The target cells were added to the effector cells at 1×10^4 cells $well^{-1}$ to give effector:target ratios of 100:1, 50:1, 25:1 and 12.5:1 (unless otherwise stated) and the plates were centrifuged at 100g for 3 min in order to allow the effectors and targets to come into contact. A control plate was set up so that the spontaneous (100µl of target cells + 100µl of cRPMI) and total (100µl of targets + 100µl of 5% triton x100) isotope release could be determined. The plates were incubated for 4 hours at 37°C in 5% CO_2 in air. For initial experiments, each effector cell type was assayed using autologous and allogeneic infected and mock-infected target cells. At the end of the incubation period the plates were centrifuged at 100g for 3 min and 100µl of supernatant was removed from each well. The supernatants from the assay and the control plates were counted in a gamma counter (Philips PW4000). The background mean counts per minute (cpm), obtained by counting 24 empty tubes prior to counting the assay samples, was subtracted from each experimental and control value.

The % specific cytotoxicity (corrected to take account of the background cpm) for each

well was calculated using the following formula:

(cpm sample-cpm spontaneous)/(cpm total-cpm spontaneous) x 100

The data were analyzed and reduced using a computer spreadsheet package (Lotus 1-2-3) which calculated the % spontaneous release of chromium from the target cell, the individual % specific cytotoxicity values for each well, the mean % specific cytotoxicity for each E:T ratio and the standard deviation using a replicate number of 4.

Significant differences between individual mean % specific cytotoxicity values were determined using the Student's *t* test.

3.2.4. Inhibition of specific killing using cold targets.

Infected or mock-infected unlabelled target cells from autologous or MHC mis-matched animals were tested for their ability to inhibit EHV-1 specific CTL activity by adding them in excess to the assay wells.

3.2.5. Time course of EHV-1 specific CTL induction

This experiment was designed to determine the effect of induction culture duration on the development of EHV-1 specific CTL. CTL induction cultures were set up 10, 8, 6, 4 and 2 days before carrying out a CTL assay. The protocol described above (see 3.2.3.) was used except that the cultures were scaled down to T25 tissue culture flasks. The total culture volumes were 10ml each containing 0.5ml of Ab4/9 ($1 \times 10^{7.6}$ $TCID_{50}ml^{-1}$) or mock supernatant. The experiment was carried out on two EHV-1 immune horses which were

equine leucocyte antigen (ELA) class I mis-matched as determined by iso-electric focusing of immuno-precipitated class I proteins derived from PBMC. All effector populations were assayed simultaneously using infected and mock-infected target cells from both animals, thus giving results for autologous and allogeneic killing.

3.2.6. The effect of virus concentration on CTL induction

The effect of varying the amount of live EHV-1 strain Ab4 in the CTL induction culture was assessed in two experiments. In the first experiment, the amount of EHV-1 was titrated from $3.6x10^5$ $TCID_{50}ml^{-1}$ to $1.75x10^8$ $TCID_{50}ml^{-1}$. In the second experiment the amount of EHV-1 was titrated from $4.0x10^2$ $TCID_{50}ml^{-1}$ to $1.0x10^6$ $TCID_{50}$ ml^{-1}. The assays were performed as described in section 3.2.2. using a total culture volume of 10ml.

3.2.7. Cross reactivity of EHV induced CTL

In order to assess the extent of cross-reactivity of *in vitro* induced CTL, induction cultures were set up with either EHV-1 strain Ab4/9, EHV-1 strain Ab4/13 cloned (Glasgow) or EHV-4 strain MD. The individual virus isolates were adjusted in order to provide an equivalent $TCID_{50}$ in each induction culture. As EHV-4 was found not to infect PWM blast cells to a sufficiently high level for target cell production (see chapter 2), all induced effector cells were assayed on EHV-1 strain Ab4-infected and mock-infected targets. Cross-reactive cytotoxic effector cells were induced from PBMC of a number of conventional and SPF foals in order to determine whether CTL develop after a true primary infection with EHV-1.

3.2.8. The effect of rhIL2 on EHV-1 specific CTL induction

The effect of inducing EHV-1 specific CTL in the presence of rhIL2 was studied. Cytotoxic effector cells were induced from PBMC of two ELA mis-matched horses in the presence of 200 $IUml^{-1}$, 20 $IUml^{-1}$, 2 $IUml^{-1}$ and 0 $IUml^{-1}$ of rhIL2. The resultant CTL were assayed on infected and mock-infected autologous and allogeneic target cells.

3.2.9. Depletion of T cell sub-sets

In order to determine the phenotype of the cells responsible for the CTL activity in EHV-1 specific CTL effector cell cultures, assays were performed using effector populations depleted of cells expressing either $CD4^+$ or $CD8^+$ on the cell surface.

Effector CTL were induced in bulk culture using live EHV-1 for six days as described above. The cells were harvested and the number of viable cells was determined using trypan blue exclusion. Aliquots of 1.5×10^7 viable effector cells were labelled with a 1/500 dilution of monoclonal antibodies specific for either equine CD4 (HB61A, VMRD inc.), equine CD8 (HT14A, VMRD inc.) or equine thymocytes (EqT6, VMRD inc.), on ice for 15 min. The equine thymocyte antibody was included as a negative control as it has been shown not to bind to equine PBMC. The cells were washed twice in cold HBSS + 10% FCS and resuspended in 180µl of medium to which was added 20µl of MACS colloidal super-paramagnetic microbeads conjugated with goat anti-mouse immunoglobulin G (Miltenyi Biotec GmbH, Germany). The cells were incubated at 4°C for 15 min and then applied to a pre-washed mini MACS column (Miltenyi Biotec

GmbH, Germany). The mini MACS system is a method for separating cells which have been labelled with very small (100nm diameter) super-paramagnetic microparticles. After labelling, the cells are applied to high gradient magnetic (HGM) columns which bind the labelled cell population (Miltenyi *et. al.* 1990). The non-bound cells were pelleted and applied to another mini MACS column in order to increase the efficiency of the separation. The resultant effector CTL were washed and used in a standard EHV-1 CTL assay using infected and mock-infected autologous and infected allogeneic target cells.

3.2.10. Viral protein specificity of anti EHV-1 CTL

In a number of herpesvirus infections, CTL are produced which are specific for immediate early (IE) proteins (Reddehase & Koszinowski, 1984 and Banks *et. al.*, 1993). The specificity of the CTL expressed at different stages of infection was assessed in an experiment based on the selective inhibition of EHV-1 proteins expressed on target cells. The method, based on that used by Tigges *et. al.* (1992), compared the specific cytotoxic activity of effectors against drug-treated and untreated, infected and mock-infected target cells. Target PWM blast cells (2.5×10^6 cells) were treated with $25 \mu g ml^{-1}$ cycloheximide (Sigma) for one hour and then infected with EHV-1 strain Ab4/9 at a moi of 10 in serum-free RPMI containing $20 IU ml^{-1}$ rhIL2 (Chiron) and $25 \mu g ml^{-1}$ cycloheximide for four hours. The cells were then washed in HBSS containing $5 \mu g ml^{-1}$ actimomycin D (Sigma) and labelled for 90 min with ^{51}Chromium in 200µl cRPMI containing $10 \mu g ml^{-1}$ actimomycin D and rhIL2. The treated and untreated target cells were then washed 3

times in HBSS and added to effector cells in 96 well round bottomed microtitre plates. The treated target cells were added to the CTL assay in medium containing $20\mu gml^{-1}$ Actinomycin D. The CTL assay was carried out as described above.

3.3. Results

3.3.1. Time course of EHV-1 specific CTL induction

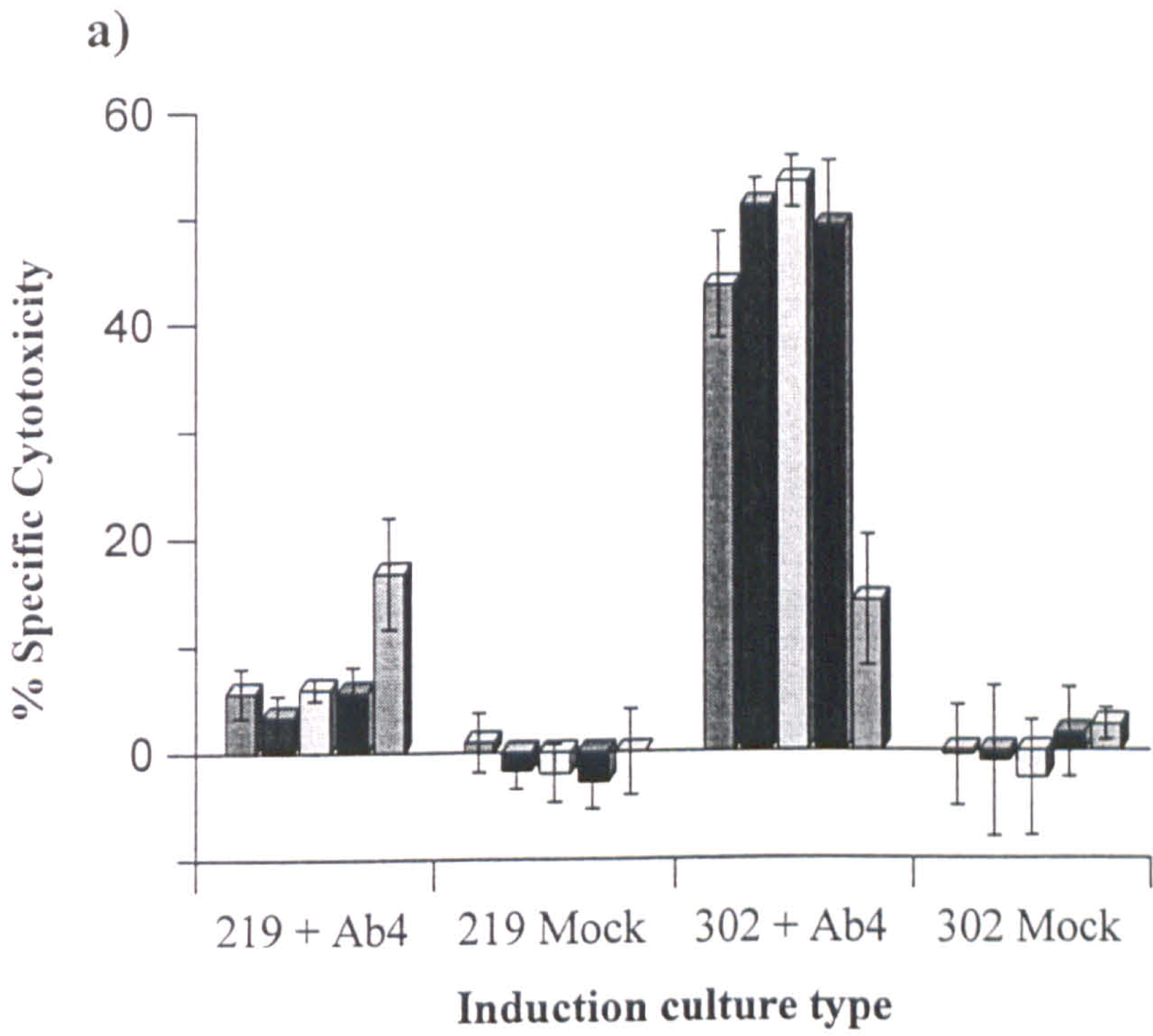
Using ^{51}Chromium release assays, it is vital to know at what time after the initiation of the induction culture the CTL activity is maximal. The results from the EHV-1 CTL time course experiment described in section 3.2. can be seen in Figures 3.1 and 3.2. Analysis of the CTL activity in PBMC from horse #302 on autologous infected target cells (Fig. 3.1) revealed that specific cytotoxicity significantly above background was not detectable until the PBMC were cultured with live EHV-1 for 4 days. Culture times longer than 4 days (up to a maximum of 10 days) resulted in very similar specific release values (around 50%) when the effector cells were assayed on autologous infected target cells. This high level of cytotoxicity was maintained when the effector cells were assayed at an effector:target ratio of 50:1 (Fig. 3.1b). A reduction in CTL activity in cultures incubated for 10 days was seen. When CTL from horse #302 were assayed on allogeneic infected target cells (i.e. those from the mis-matched horse #219) the specific cytotoxicity increased as the induction culture time increased (Fig. 3.2). This indicated that, in this particular animal, increasing the induction culture time resulted in the appearence of an

Figure 3.1. Time course of EHV-1 strain Ab4 specific CTL induction.

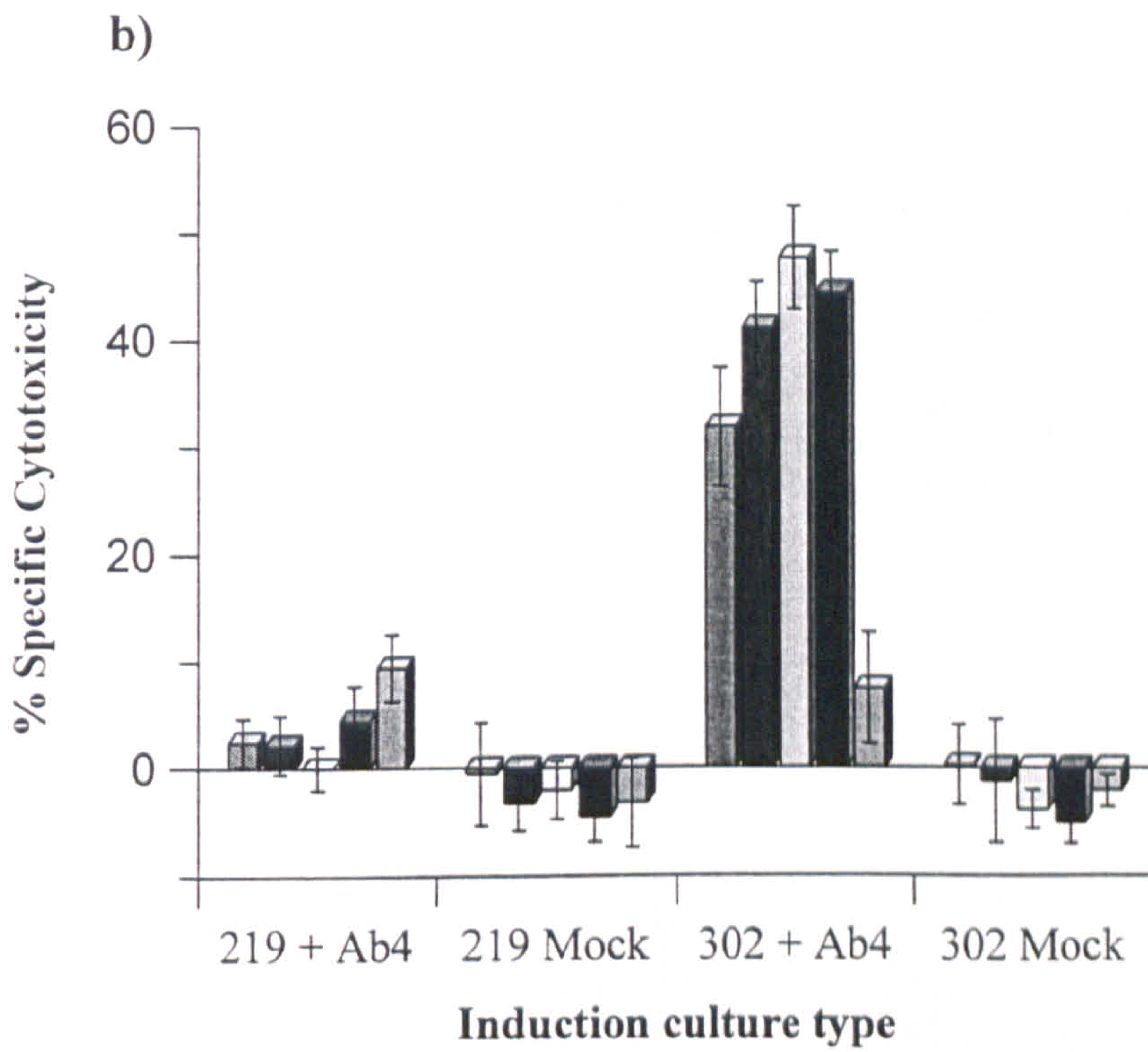
Mare #302 EHV-1 infected targets.

a) Effector:Target ratio = 100:1.
b) Effector:Target ratio = 50:1.

a)



b)



Time in culture:

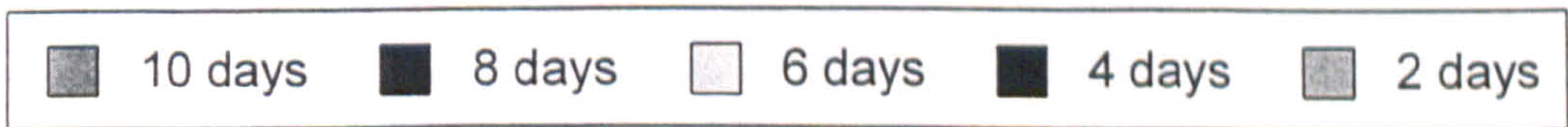
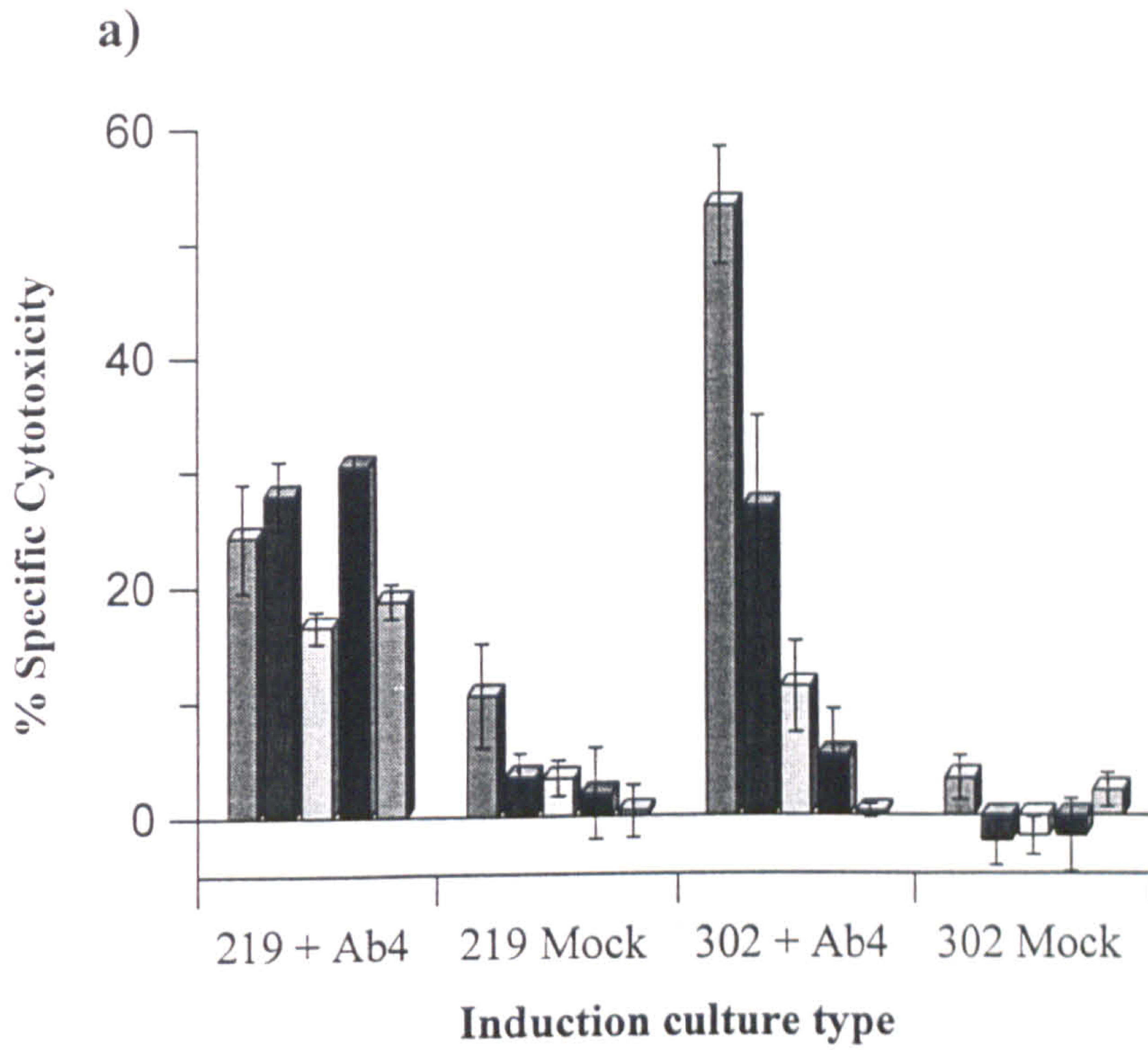


Figure 3.2. Time course of EHV-1 strain Ab4 specific CTL induction.

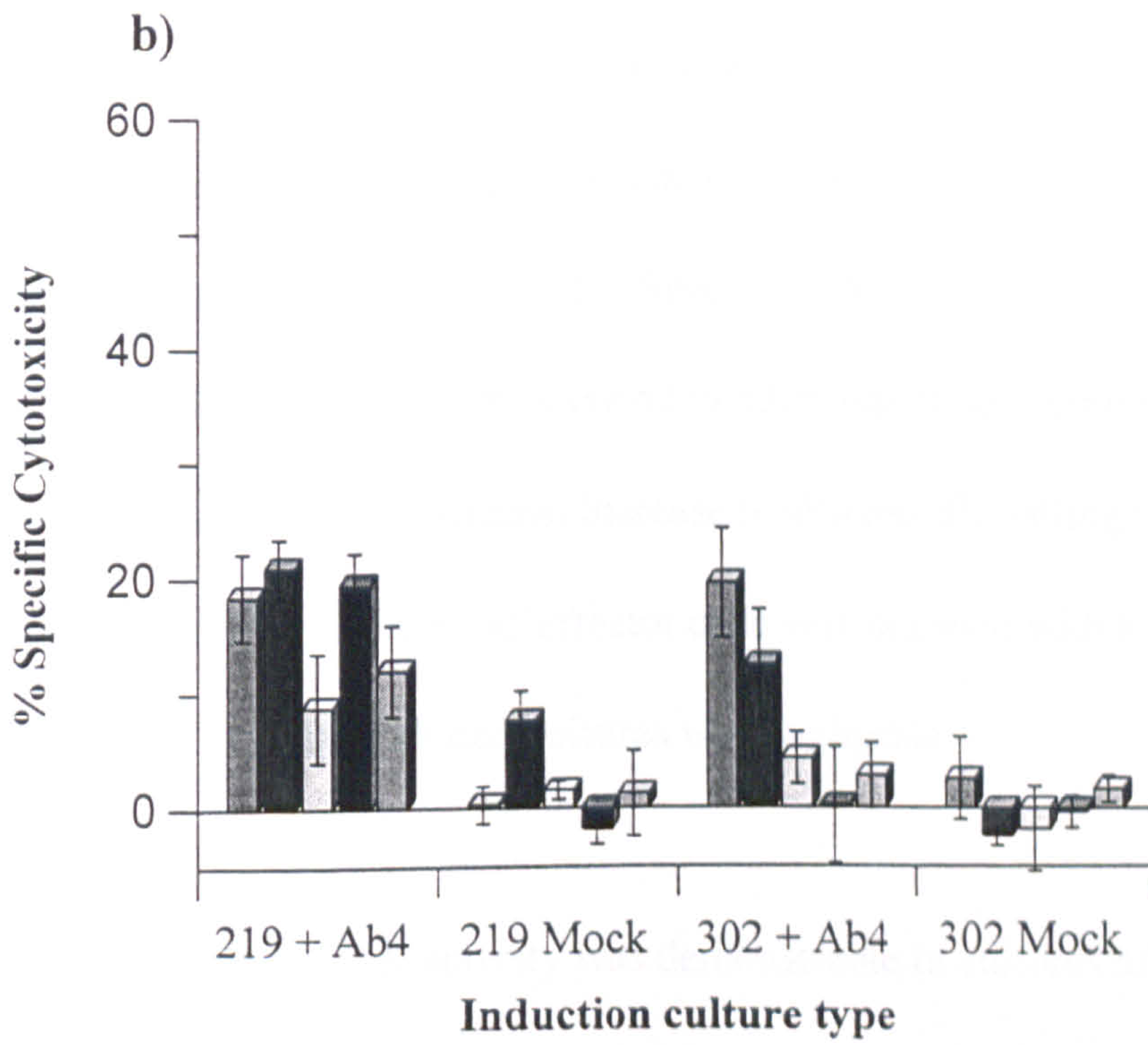
Mare #219 infected targets.

a) Effector:Target ratio = 100:1.
b) Effector:Target ratio = 50:1.

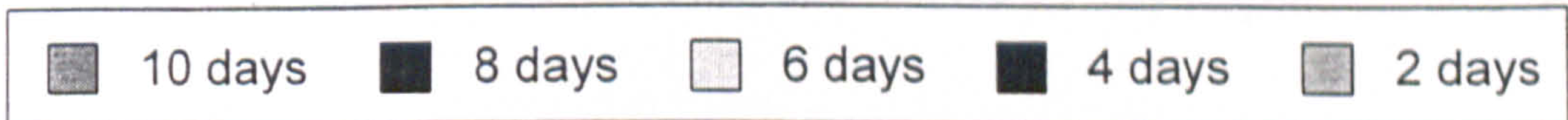
a)



b)



Time in culture:



additional population of non-genetically restricted cells. The cytotoxicity due to this additional population of cells was reduced when the effectors were assayed at an effector:target ratio of 50:1 (Fig. 3.2b). Cytotoxicity against both sets of target cells due to mock-induced PBMC from #302 was negligible indicating that the CTL activity was EHV-1 specific.

The results obtained using PBMC from #219 as effectors showed that the maximal specific cytotoxicity value using autologous infected target cells (Fig. 3.2) was lower than that obtained with #302 effector cells. This was a consistent result when measuring CTL from these two animals. Significant cytotoxicity was demonstrable against autologous infected target cells by effector cells from horse #219 after 2 days induction. This CTL activity was, however, not genetically restricted as similar cytotoxicity was detected using allogeneic target cells (see Fig. 3.1). Specific cytotoxicity above background was measurable using effector CTL from horse #219 after induction cultures were induced for between 4 and 10 days. The significant increase in allospecific killing with increasing time in culture seen when using #302 effector cells was not seen with #219 effectors. Again, cytotoxicity in mock-induced cultures was negligible.

These results indicate that CTL activity was demonstrable in cultures of PBMC induced with live EHV-1 Ab4 for between 4 and 10 days. The difference in the pattern of response between #302 and #219 indicates that different horses may respond differently to the same stimulus.

3.3.2. The effect of virus concentration on CTL induction

The effect of increasing the amount of live EHV-1 in CTL induction cultures from $3.6x10^5$ to $1.75x10^8$ $TCID_{50}ml^{-1}$ was studied. The induction cultures and assays were carried out as described above except for the concentration of inducing virus. Figure 3.3a shows that antigen-specific genetically restricted cytotoxicity was induced at all virus concentrations tested. Killing of allogeneic infected target cells by the effector cells was minimal (Fig. 3.3b).

In a second experiment the concentration of live virus in the induction cultures was titrated from $4.0x10^2$ to $1.0x10^6$ $TCID_{50}ml^{-1}$. Significant ($p<0.05$) killing, above mock-induced effectors assayed on autologous infected targets, could be demonstrated when $1.0x10^4$ $TCID_{50}ml^{-1}$ was used in the induction culture (0.05ml of virus corresponding to an approximate moi of 0.00027). Reducing the concentration of virus below this failed to stimulate the development of EHV-1 specific CTL. Mock-induced PBMC failed to kill autologous infected target cells. The induced CTL failed to kill allogeneic infected target cells (Fig. 3.4b). These results show that the concentration of virus initially used for the routine testing of EHV-1 specific CTL activity ($2x10^6$ $TCID_{50}ml^{-1}$ see 3.2.2.) allowed the maturation of precursor CTL into effector CTL. Increasing this concentration to $2x10^7$ $TCID_{50}ml^{-1}$ did not effect the cytotoxic activity.

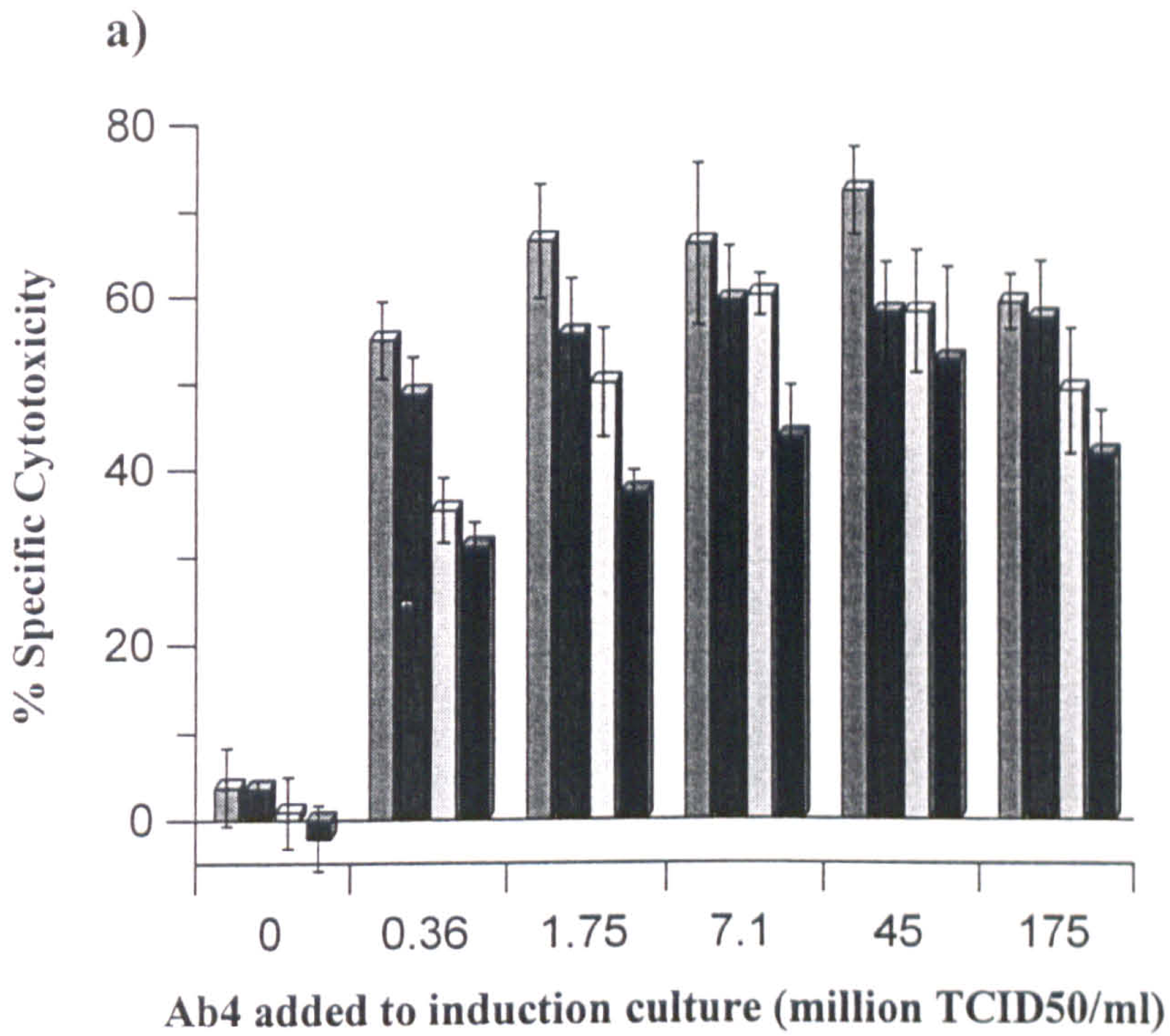
3.3.3. Inhibition of specific killing using cold targets

The inhibition of CTL killing of a labelled target cell using an excess of unlabelled "cold"

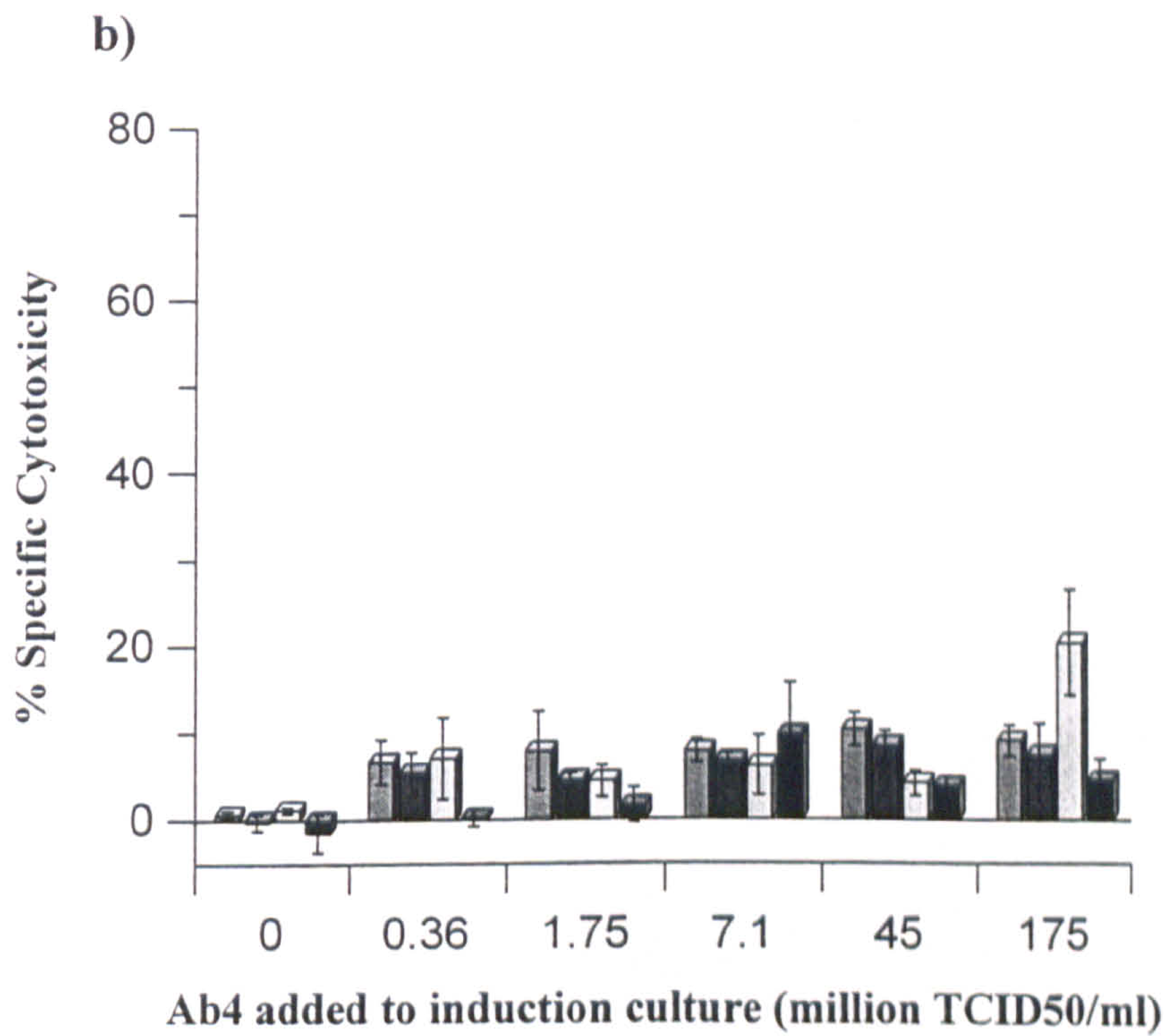
Figure 3.3. Titration of the amount of EHV-1 used in CTL induction cultures.

a) CTL from mare #302 on autologous EHV-1 infected targets.
b) CTL from mare #302 on allogeneic EHV-1 infected targets.

a)



b)



Effector:Target ratio:

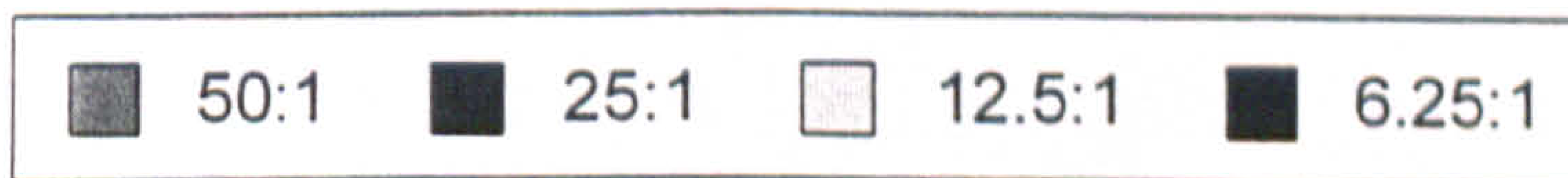
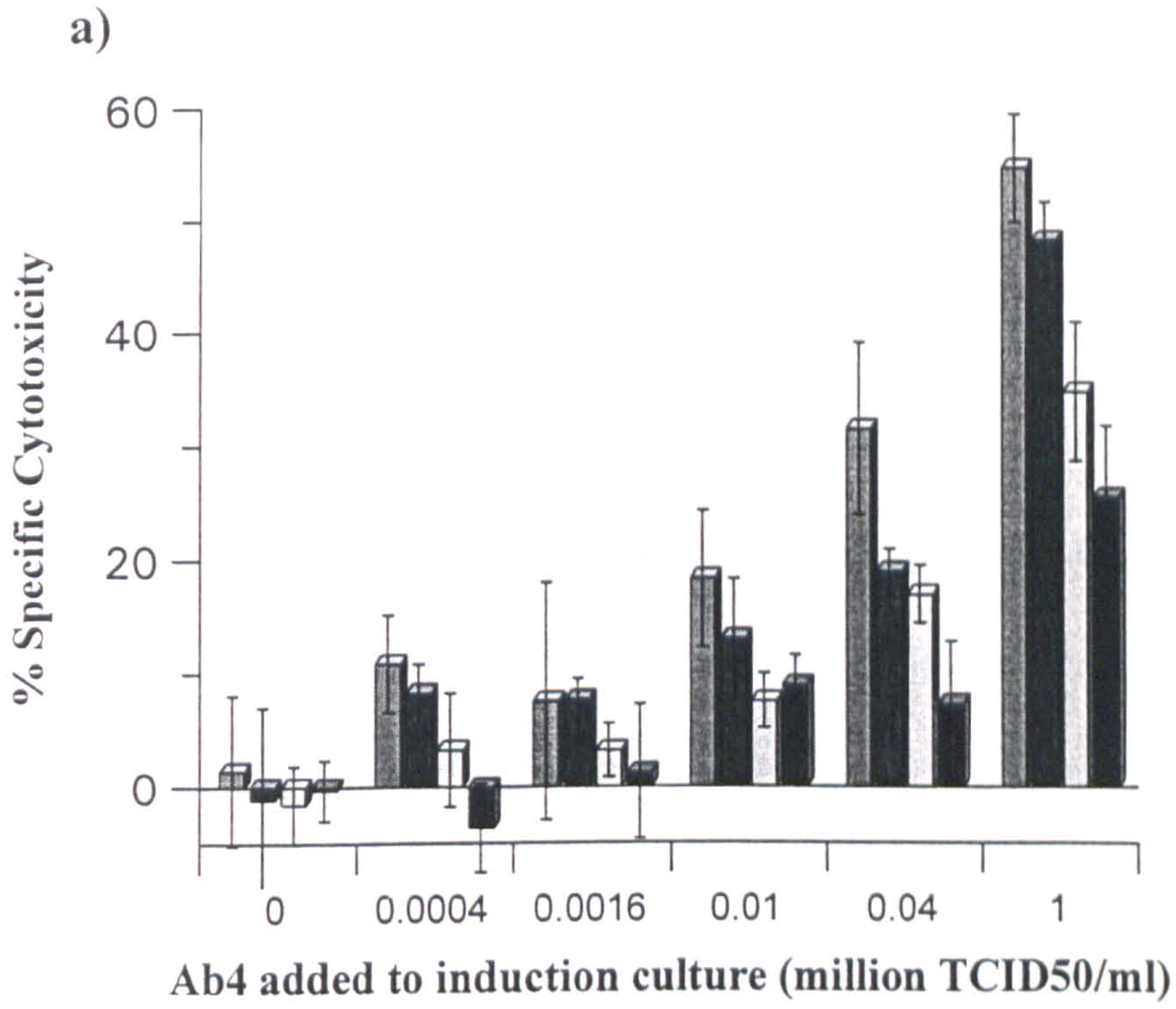


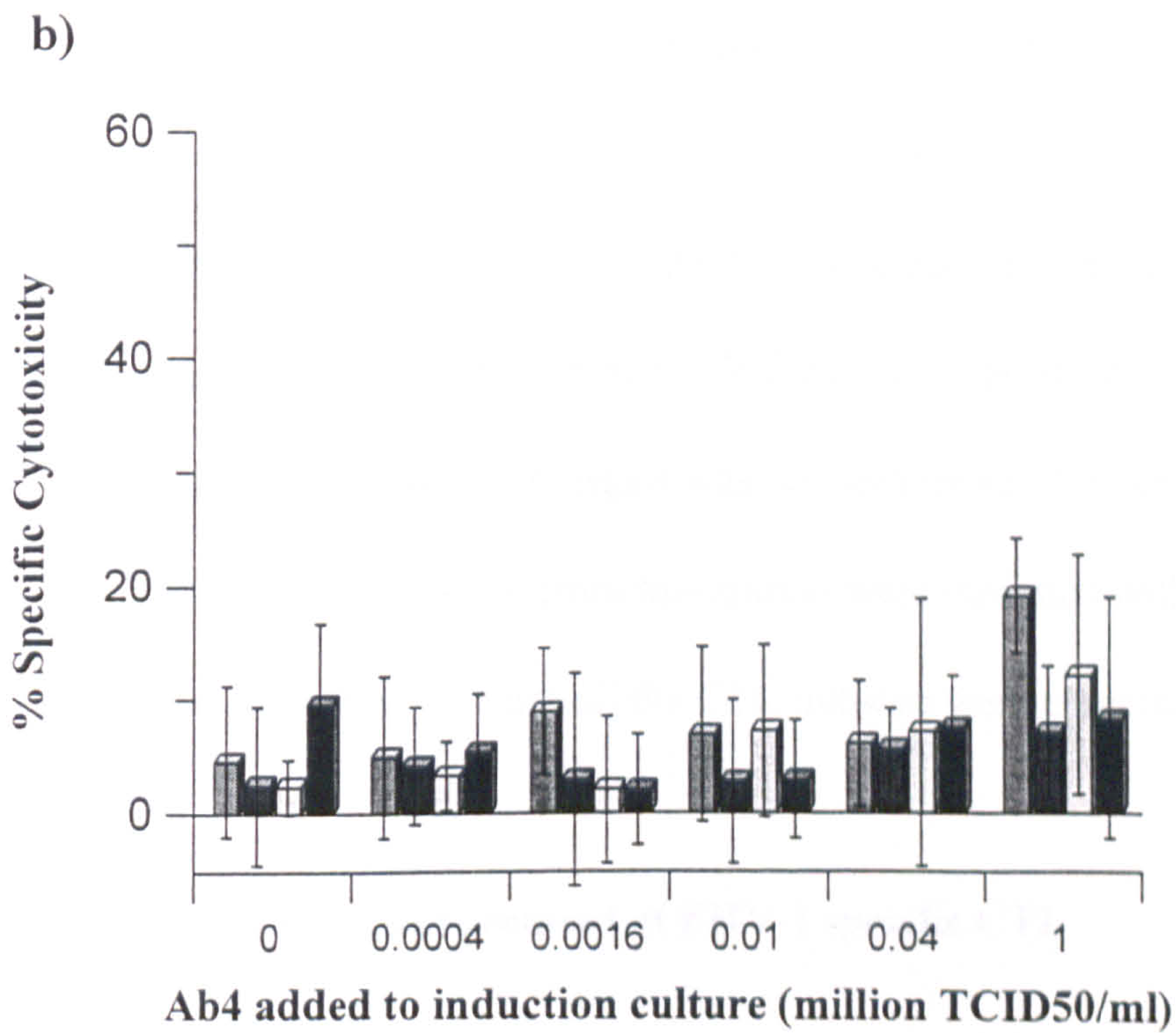
Figure 3.4. Titration of the amount of EHV-1 used in CTL induction cultures.

a) CTL from mare #302 on autologous EHV-1 infected targets.
b) CTL from mare #302 on allogeneic EHV-1 infected targets.

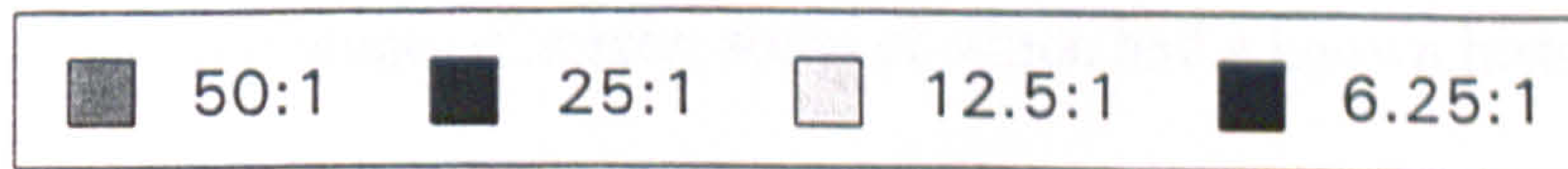
a)



b)



Effector:Target ratio:



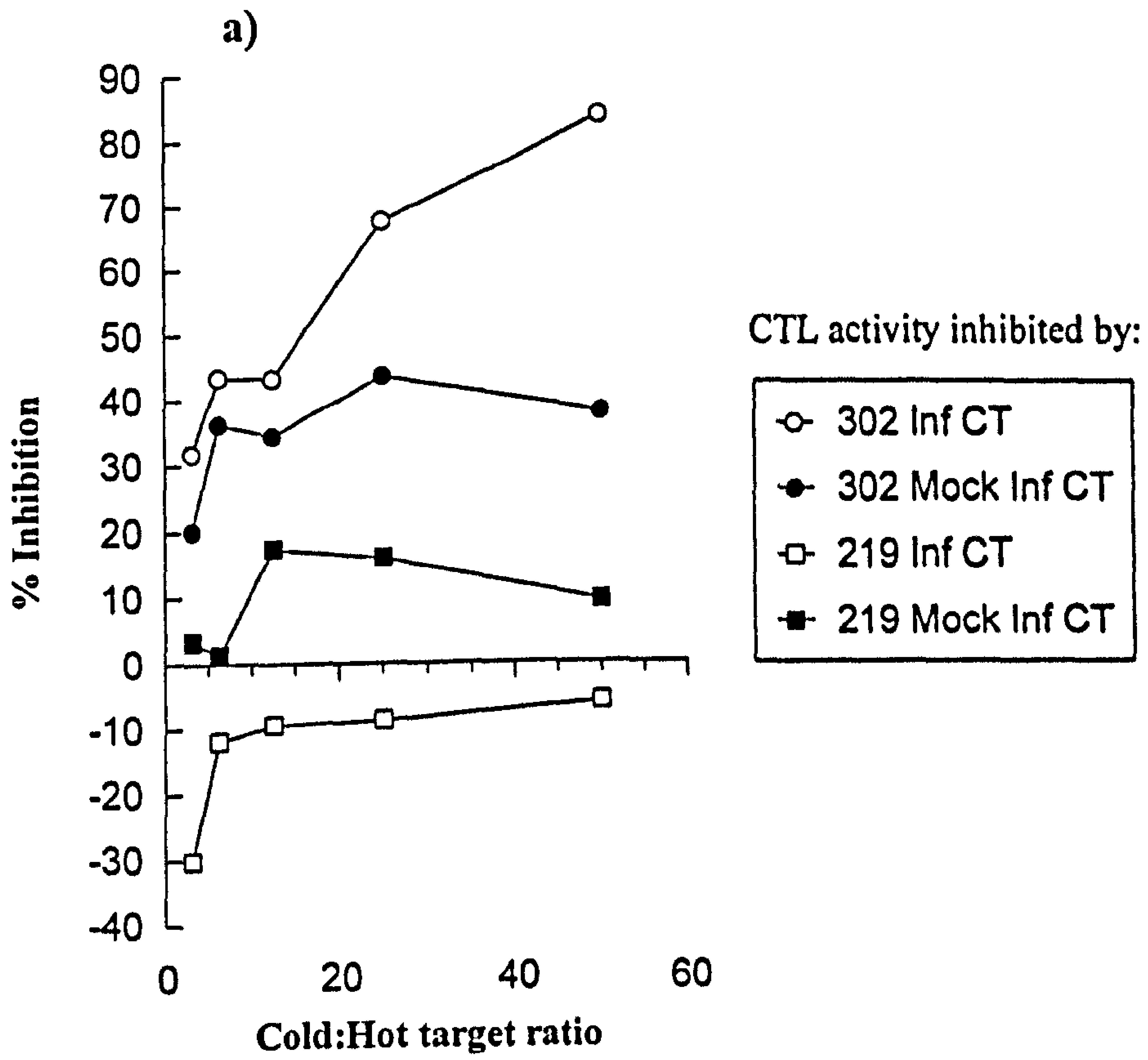
targets is an established method of evaluating the specificity of CTL. Figure 3.5 shows the % inhibition of killing seen when autologous infected (open circles), allogeneic infected (open squares), autologous mock-infected (closed circles) and allogeneic mock-infected (closed squares) cold targets were included in a standard EHV-1 specific CTL assay. The results indicated that a large percentage of the CTL activity was due to EHV-1 specific CTL as the killing was inhibited to a greater extent by autologous infected cold targets than by the autologous mock-infected cold targets. However, Fig. 3.5a also shows that there was some inhibition by autologous mock-infected cold targets. Indeed, the base line inhibition started at 20% and increased to around 40%, indicating that there was an element of non-specific cytotoxicity present in CTL effector cell populations from this particular animal. Another explanation for this inhibition was that the autologous mock-infected targets may have decreased the contact between the CTL and the labelled target cells. Inhibition of CTL activity with both infected and mock-infected allogeneic cold targets was low (below 20%) when cells from #302 were used as effectors. Inhibition of CTL activity with mock-infected cold targets was not seen using effector cells from horse #219 (see Fig. 3.5b). However, CTL from this animal were inhibited with allogeneic infected cold targets indicating that not all the CTL induced were genetically restricted.

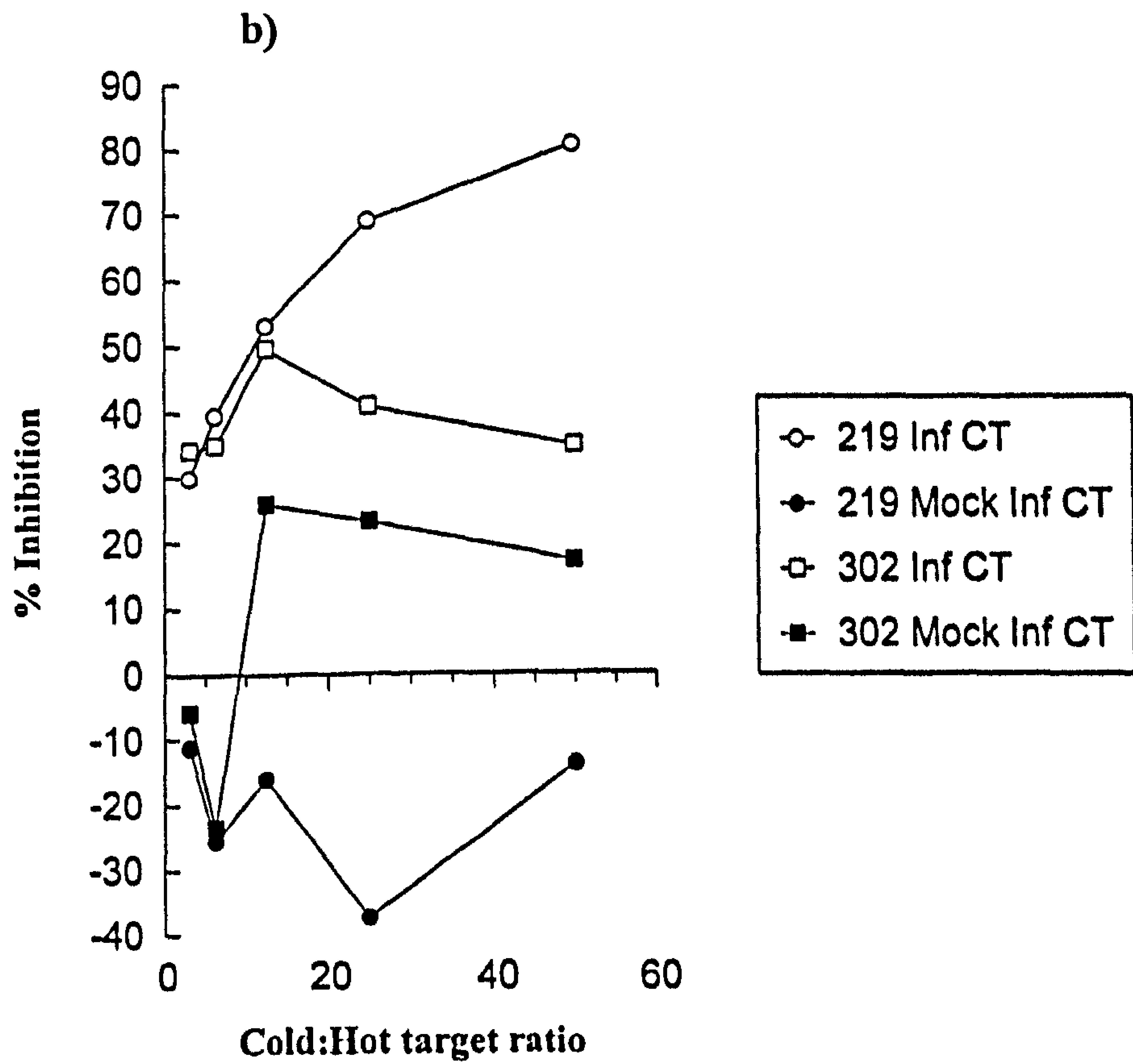
3.3.4. The generation and measurement of EHV-1 specific CTL

The method for the generation and measurement of EHV-1 specific CTL described in section 3.2. was applied to a range of horses, some of which had a known history of

Figure 3.5. Cold target inhibition of EHV-1 specific CTL.

a) Mare #302 effector cells on autologous EHV-1 infected targets.
b) Mare #219 effector cells on autologous EHV-1 infected targets.


a)
% Inhibition
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
0
20
40
60
Cold:Hot target ratio
CTL activity inhibited by:
302 Inf CT
302 Mock Inf CT
219 Inf CT
219 Mock Inf CT


b)
% Inhibition
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
0
20
40
60
Cold:Hot target ratio
219 Inf CT
219 Mock Inf CT
302 Inf CT
302 Mock Inf CT

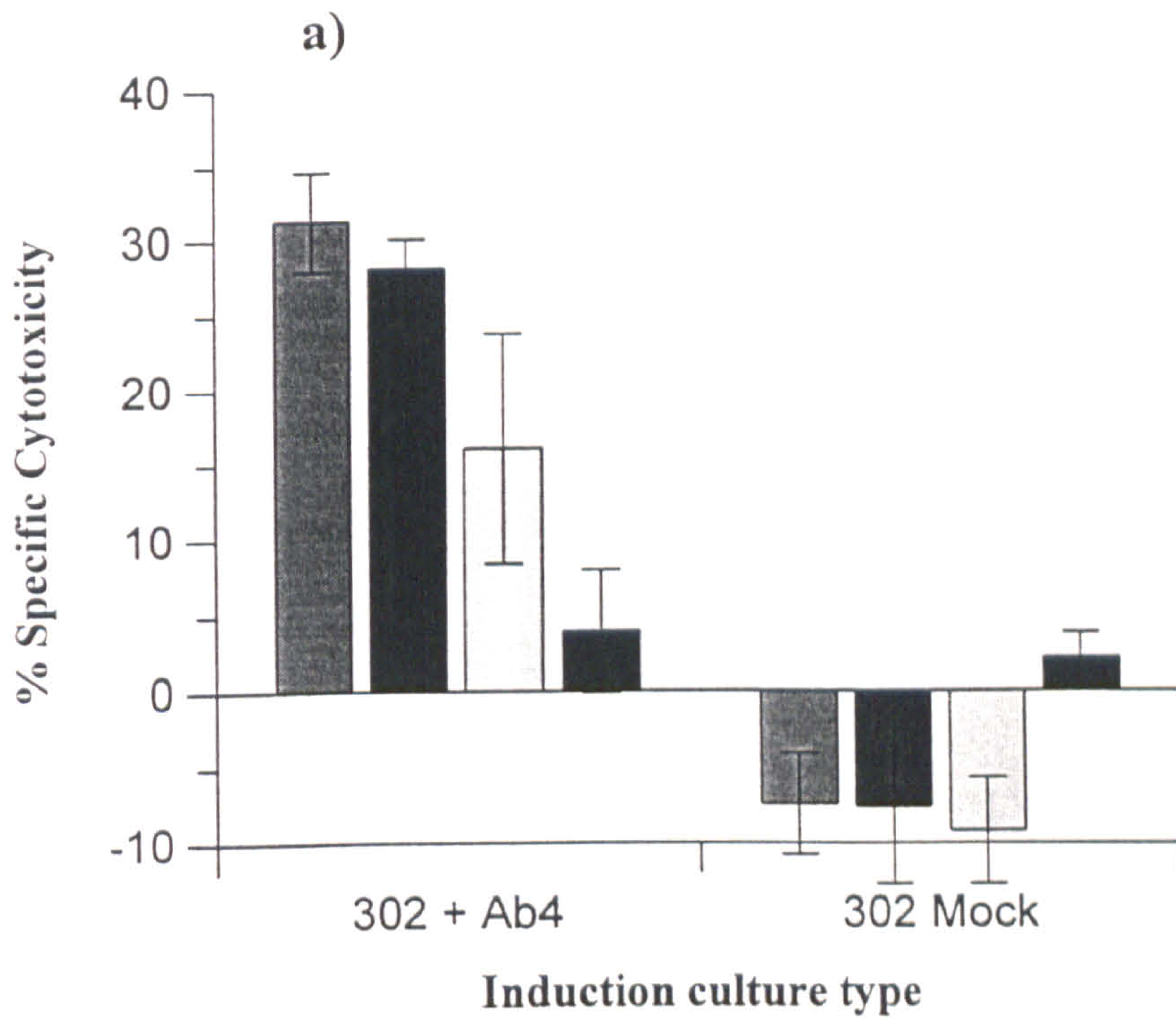
EHV-1 infection and some of which did not. In order to determine whether EHV-1 specific CTL develop after a primary infection, CTL were induced from cryopreserved PBMC derived from two SPF foals before and after infection.

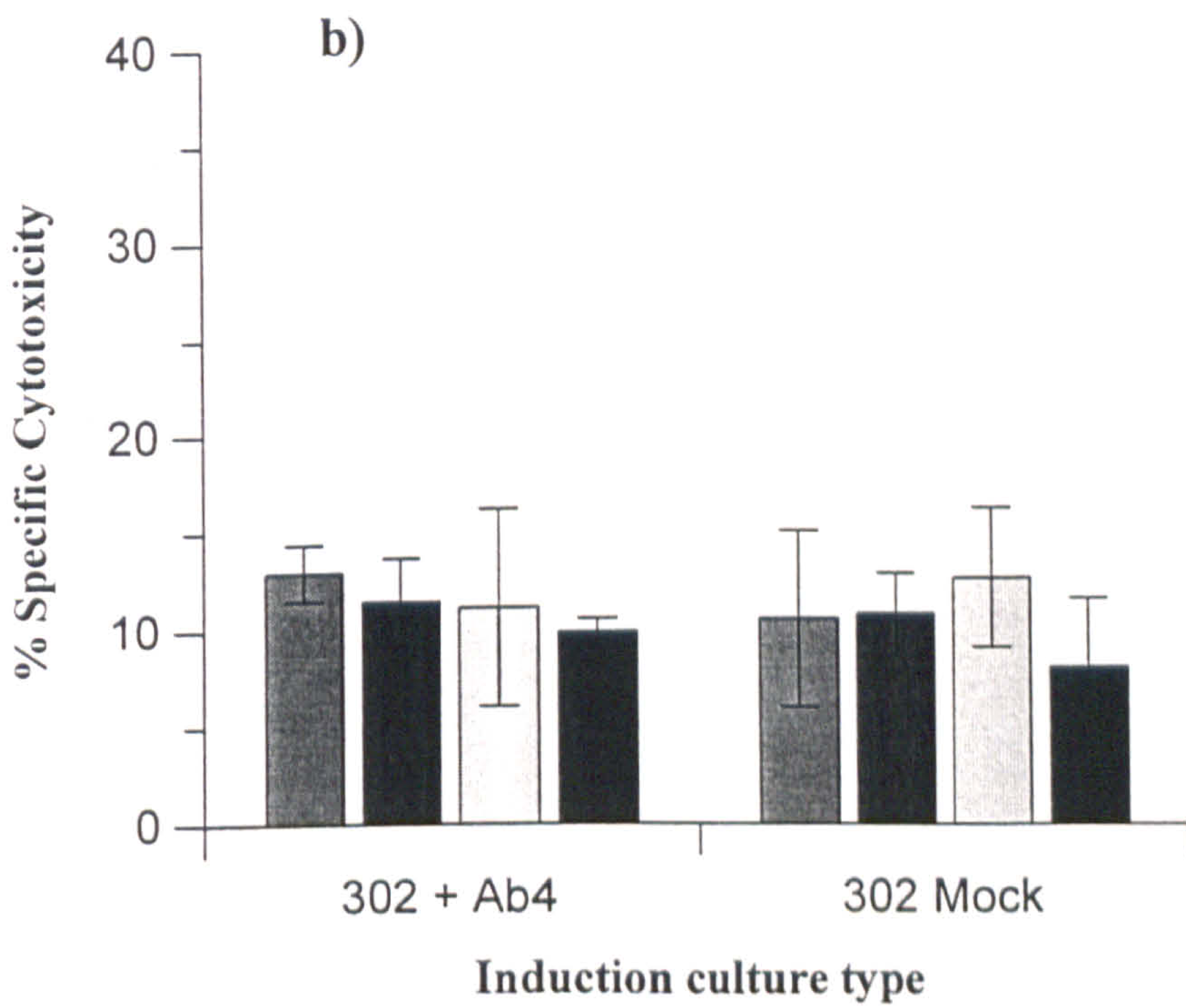
The two horses used to standardise the CTL assay in the previous section (#302 and #219) were immune animals which had experienced numerous previous experimental infections with EHV-1 (#219 received four infections and #302 received three infections within a three year period). Their CTL were EHV-1 specific and genetically restricted (Figs 3.6 and 3.7). Despite their similar infection histories, the induced CTL from these two animals were different. First, #302 consistently produced higher chromium release values although the background killing of mock-infected autologous targets was also higher (see Fig. 3.6a and 3.6b). Induced CTL from #219 consistently produced steeper dose response curves of % specific cytotoxicity against effector:target ratio than did those from #302. This may indicate a quantitative difference in frequency of CTL between the two animals and/or reflect an increased population of MHC non-restricted NK cells in the induction cultures of horse #302. Evidence for the latter comes from the relatively high level of % cytotoxicity seen when these effector cells were assayed using allogeneic infected target cells (i.e. from the ELA mis-matched horse #219). This was not seen in the reciprocal combination of effector and target cells.

Figure 3.8 shows CTL activity measured from four horses of different infection histories. The effector cells used in this experiment were either EHV-1 Ab4-induced or mock-

Figure 3.6. EHV-1 specific CTL induced from mare #302 PBMC.

a) EHV-1 infected, autologous targets.
b) Mock infected, autologous targets.





Effector:Target ratio:

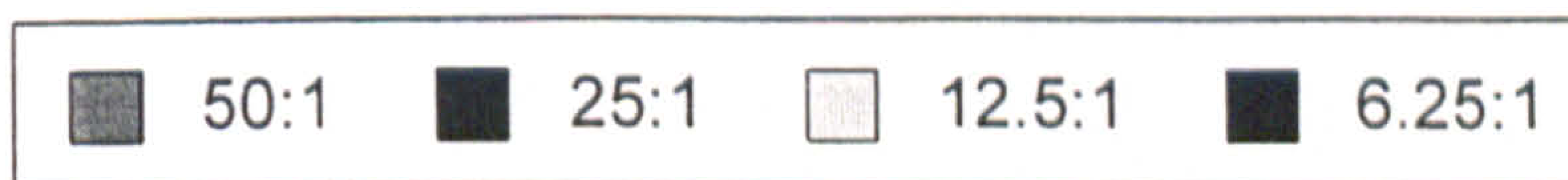
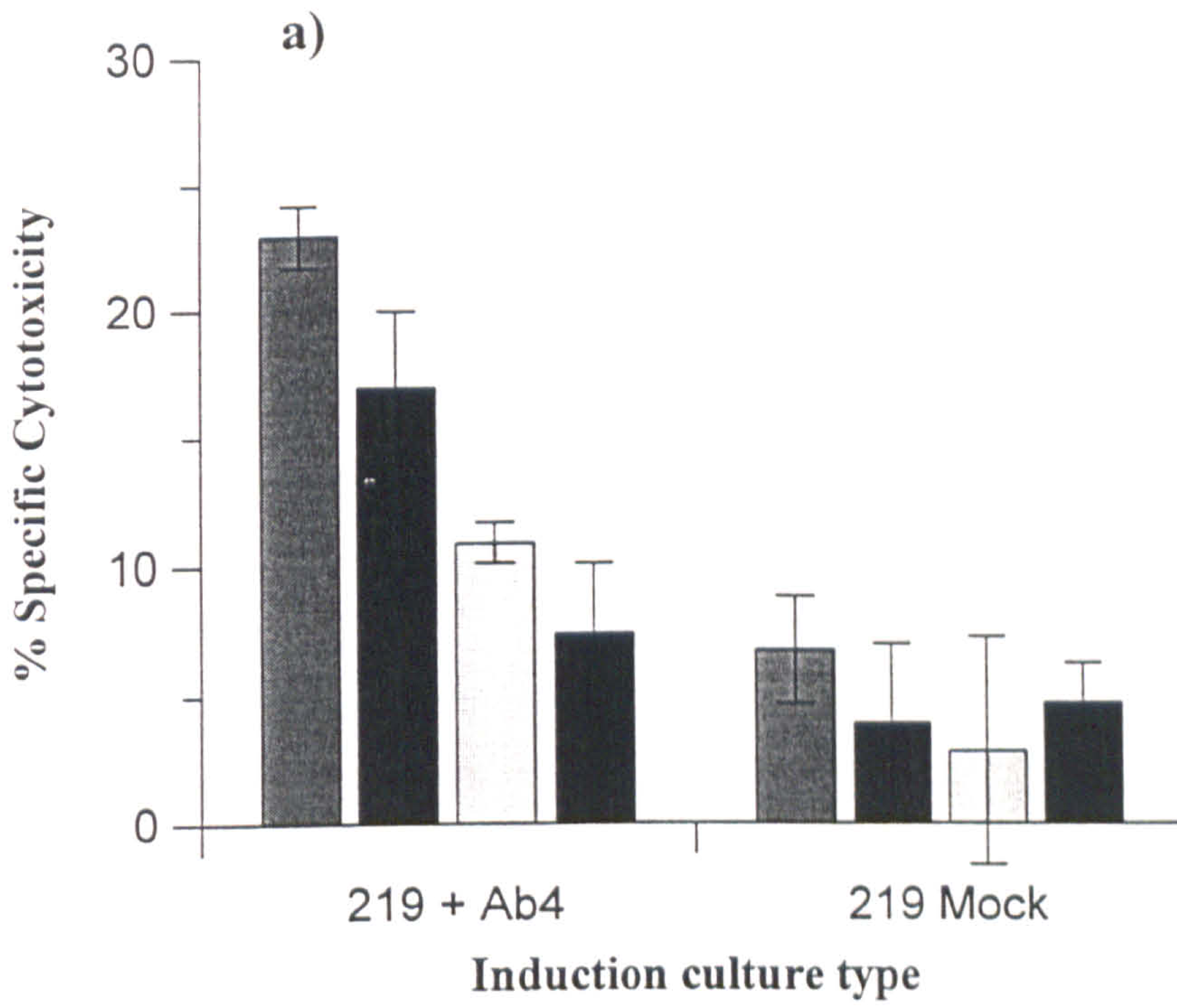
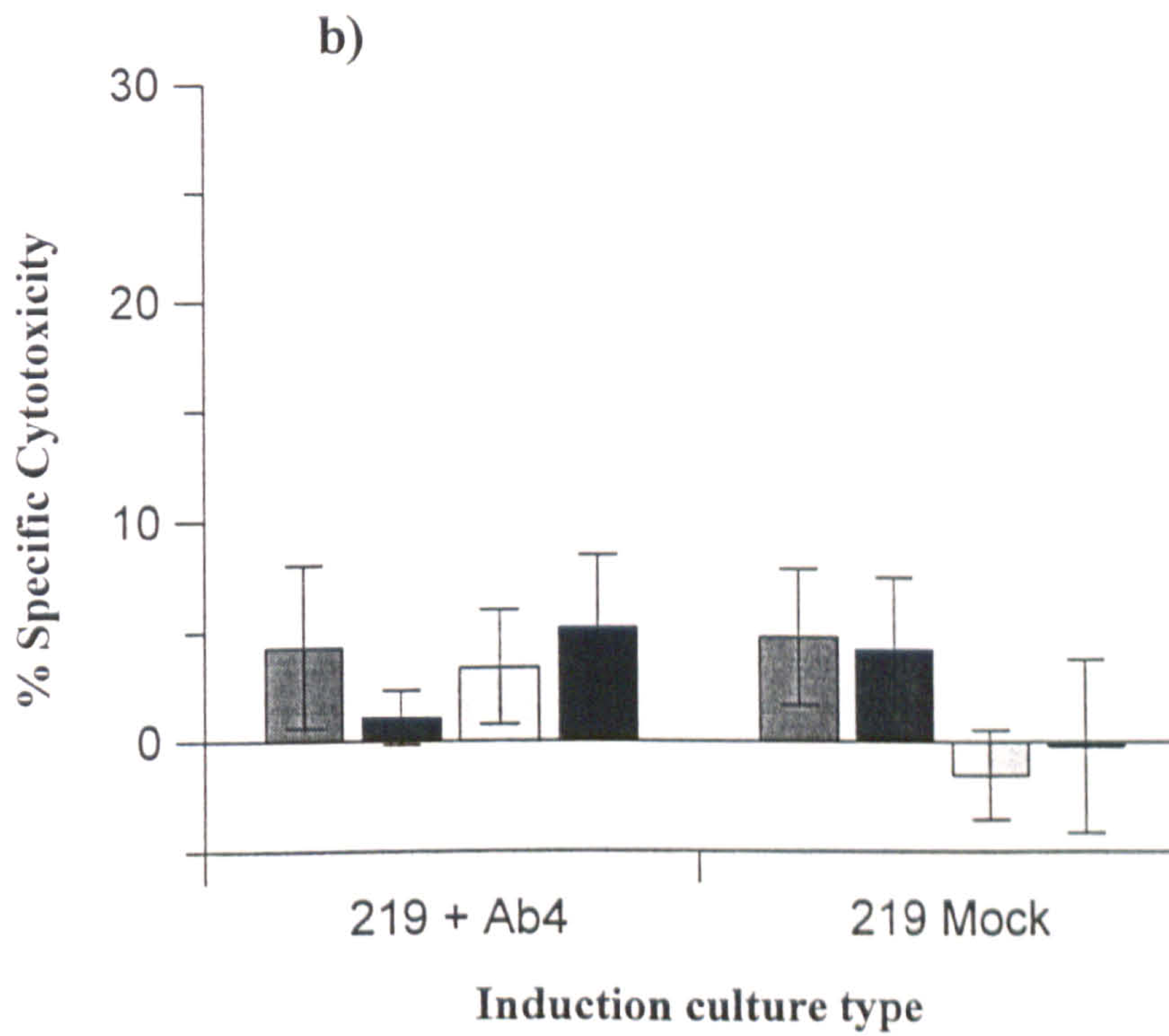


Figure 3.7. EHV-1 specific CTL induced from mare #219 PBMC.

a) EHV-1 infected, autologous targets.
b) Mock infected, autologous targets.





Effector:Target ratio:

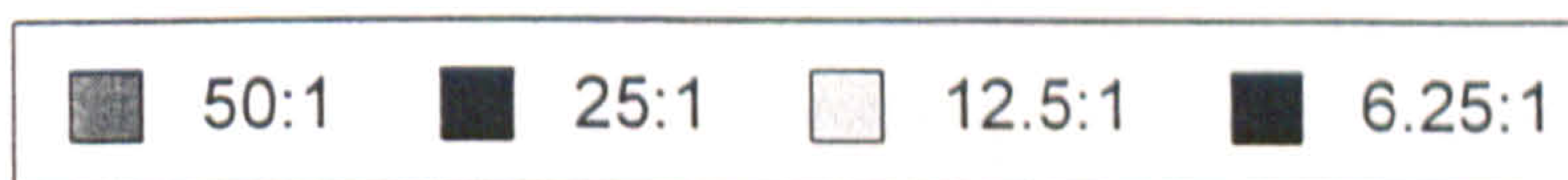
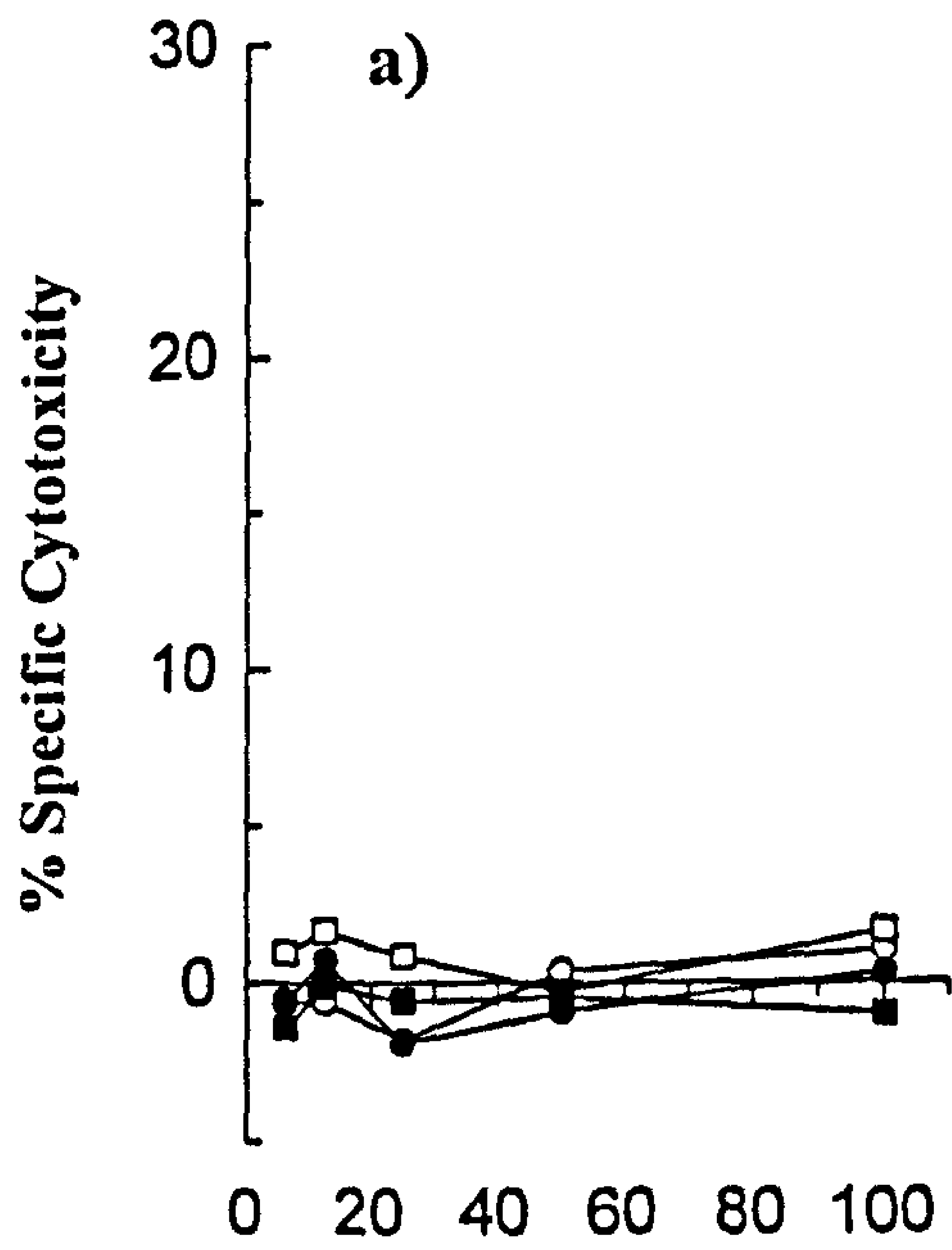
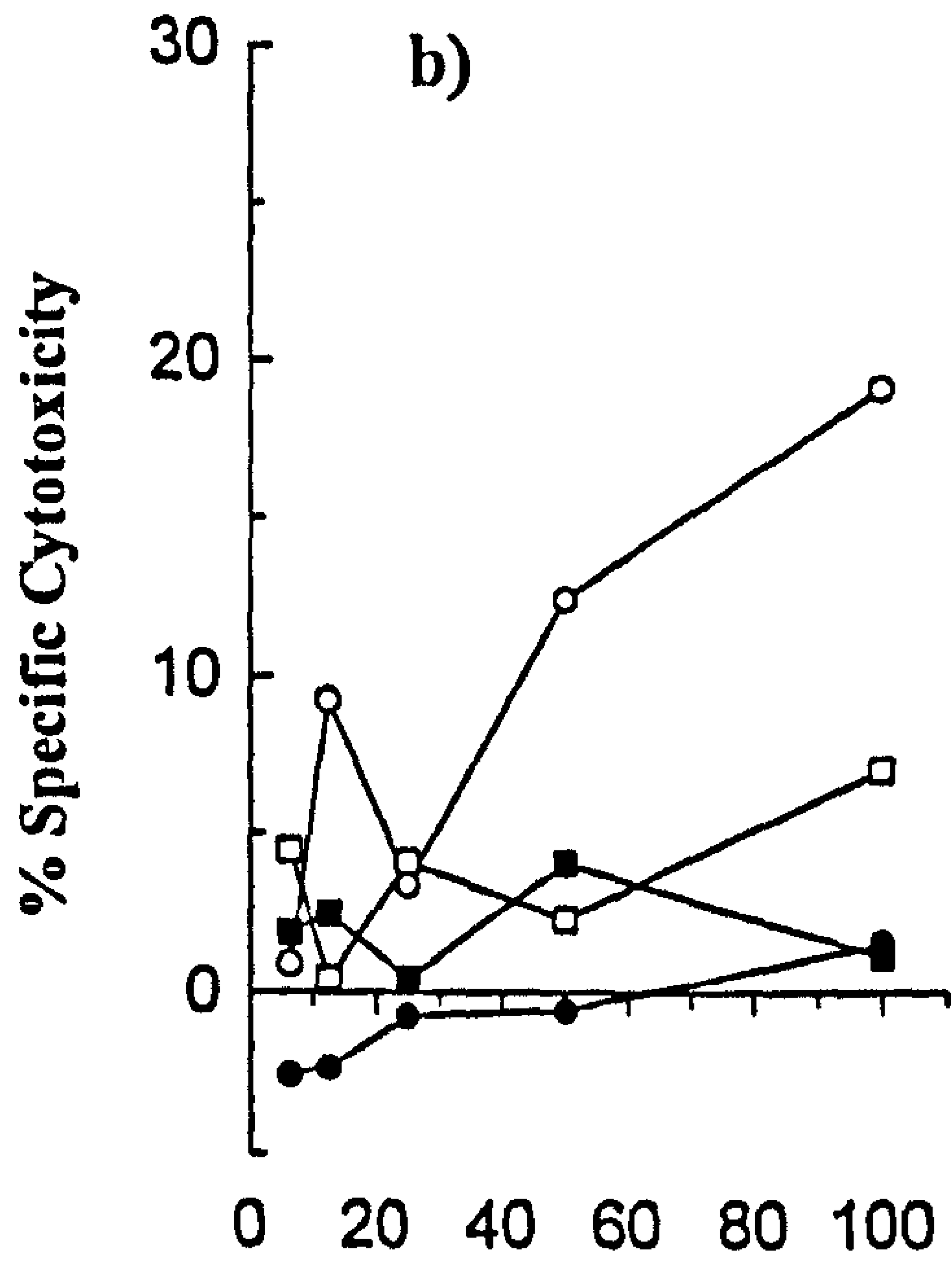
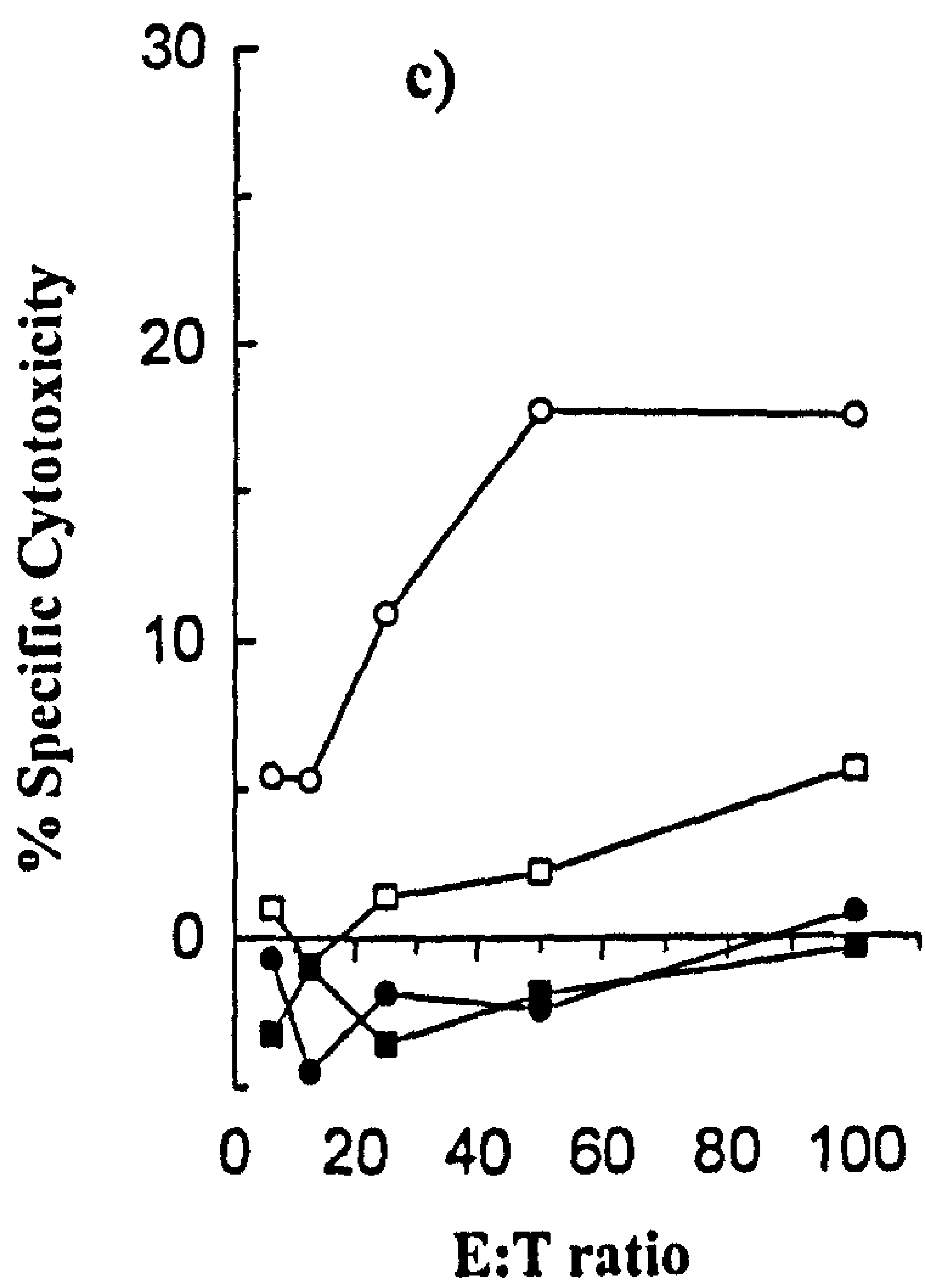


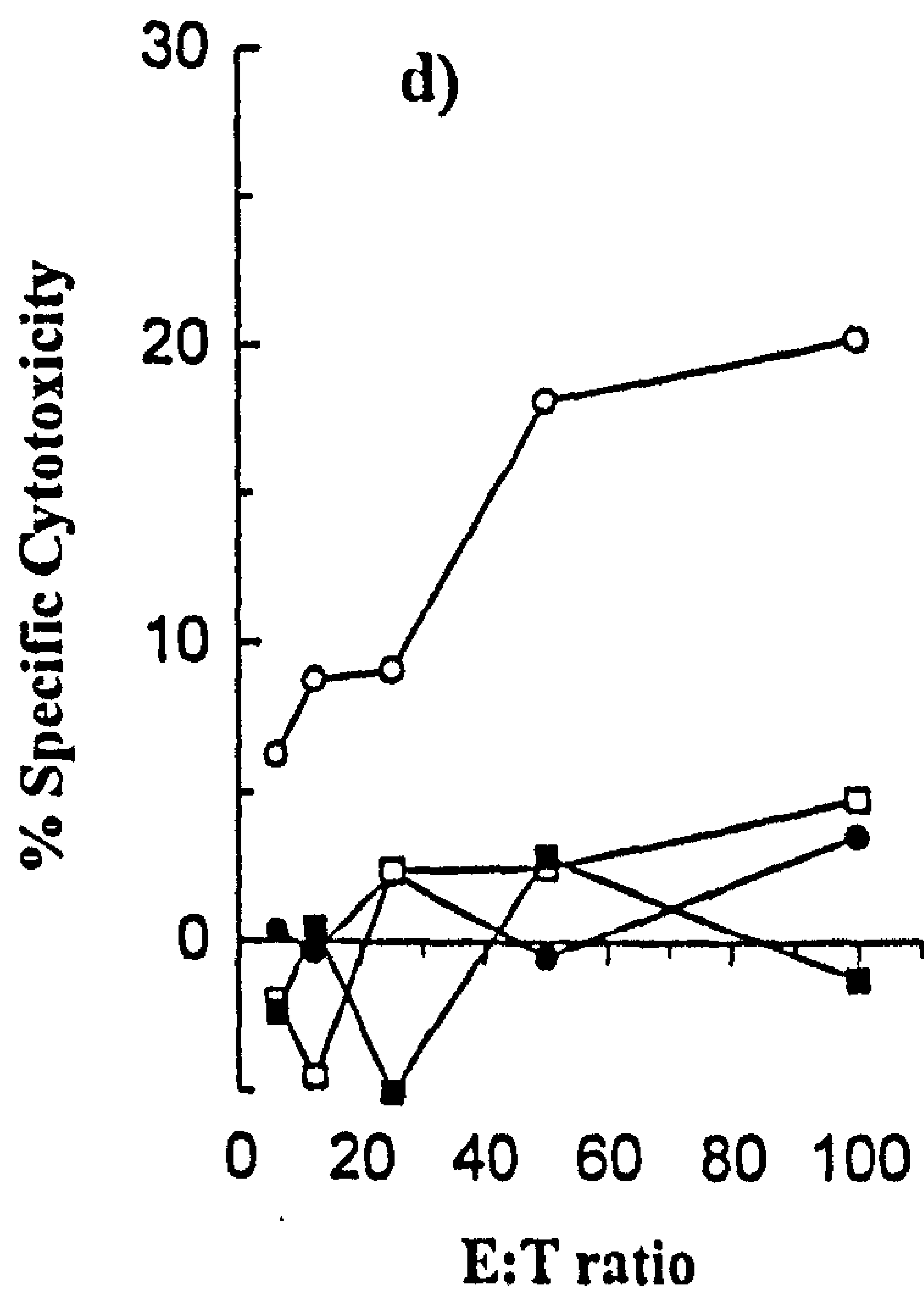
Figure 3.8. EHV-1 specific CTL from four horses of various ages.

a) Three week old foal.
b) Ten month old foal.
c) and d) Adult mares.









Induction culture/Target cell type:

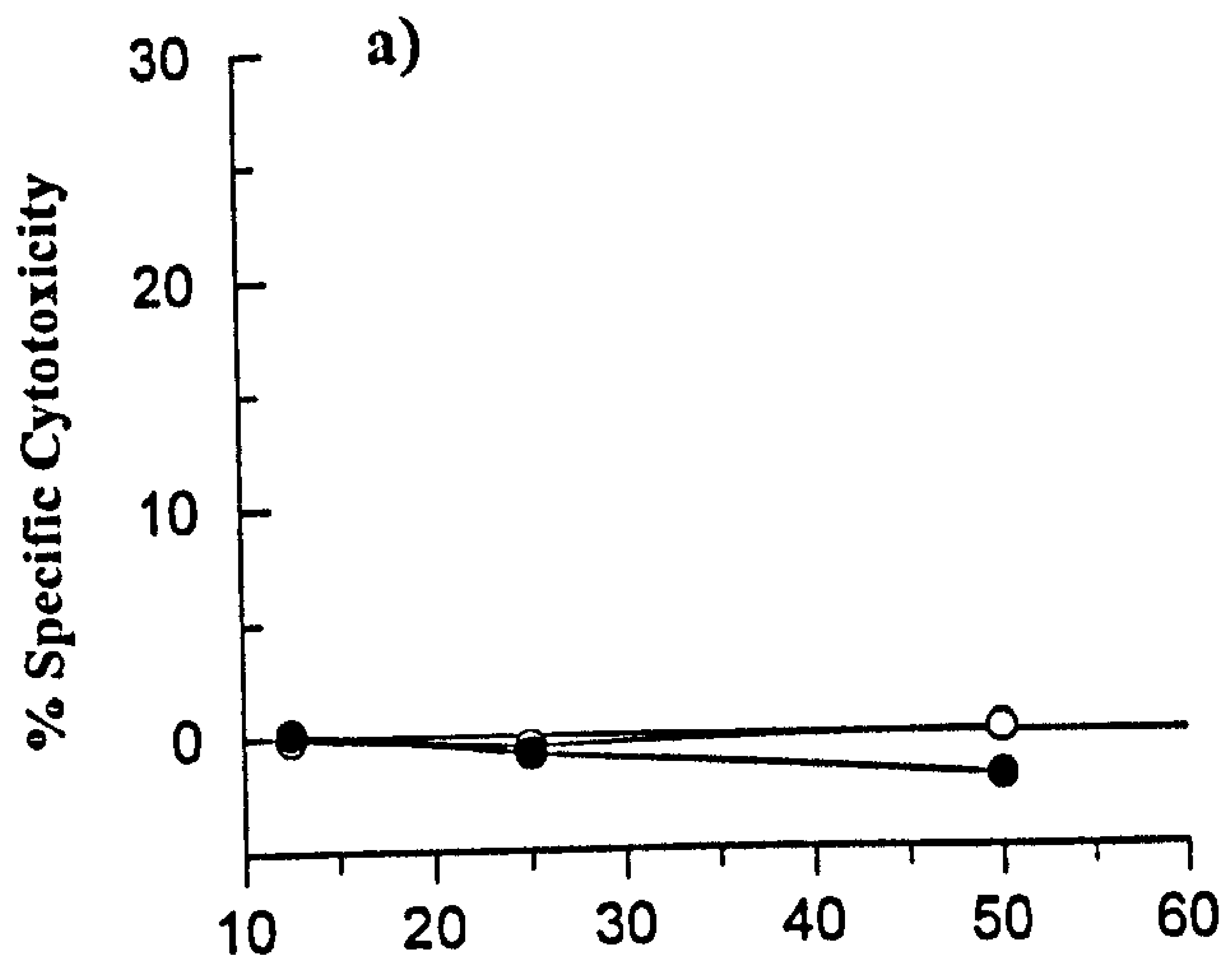
- ○ Ab4 induced/Inf. targets
- ● Mock induced/ Inf. targets
- □ Ab4 induced/Mock inf. targets
- ■ Mock induced/Mock inf. targets

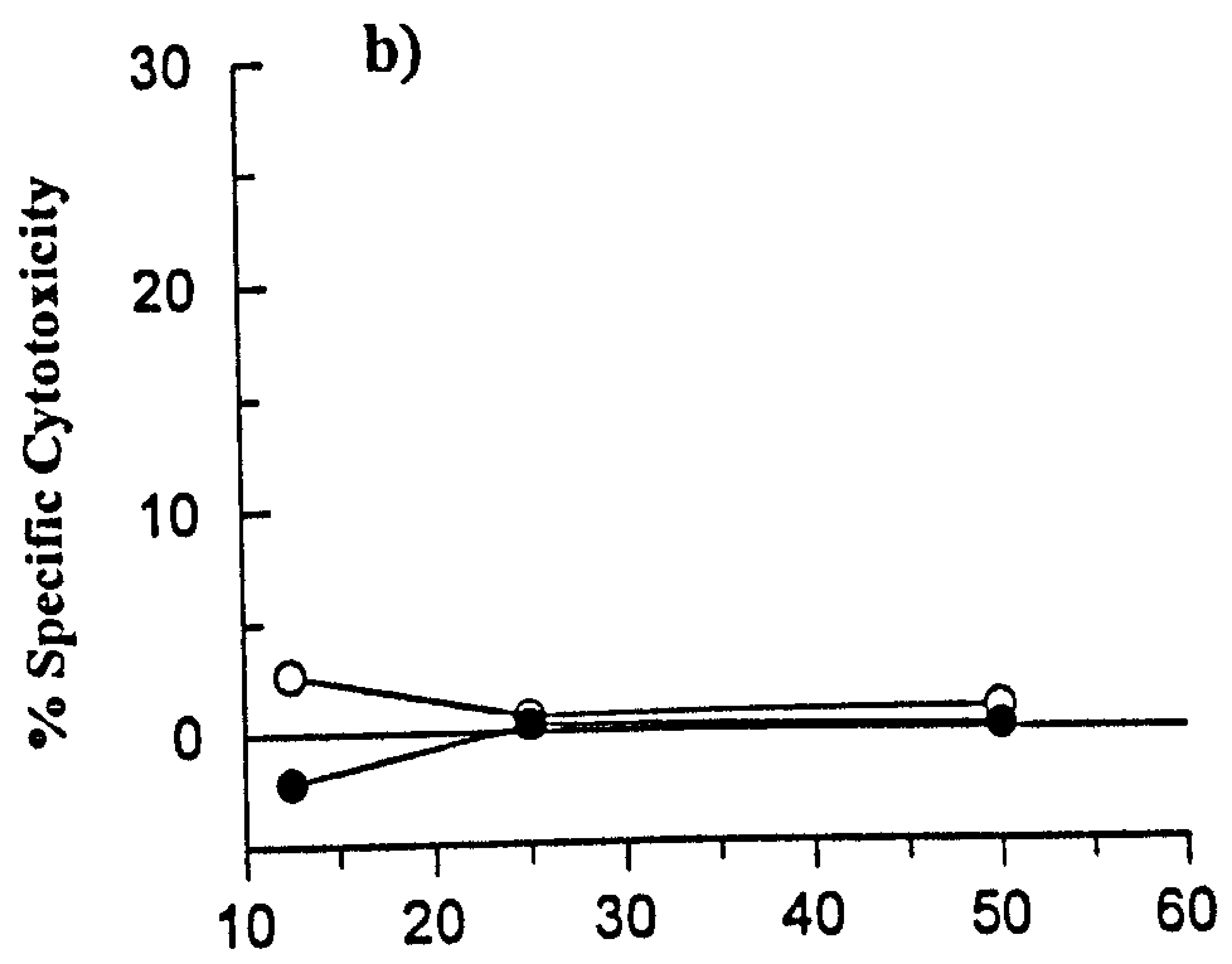
induced PBMC and CTL activity was assayed on autologous infected and mock-infected target cells. Figure 3.8a represents the CTL measured from a three-week old foal and was therefore, unlikely to have been exposed to either EHV-1 or EHV-4. Absence of detectable CTL activity was therefore not surprising. Figure 3.8b represents CTL activity from a horse which was 10 months old at the time of sampling. It is clear that this horse had experienced infection with either EHV-1 or EHV-4. The other two animals represented in Figures 3.8c and 3.8d were adult mares both of which had experienced both experimental and natural infections with EHV-1 and EHV-4. The CTL activity of cultures derived from the 10 month old animal (Fig 3.8b) decreased more rapidly at lower effector:target ratios than those from adult animals (Figs 3.8c and 3.8d). As mentioned above, this is likely to represent a difference in the frequencies of EHV-1 specific CTL between these animals.

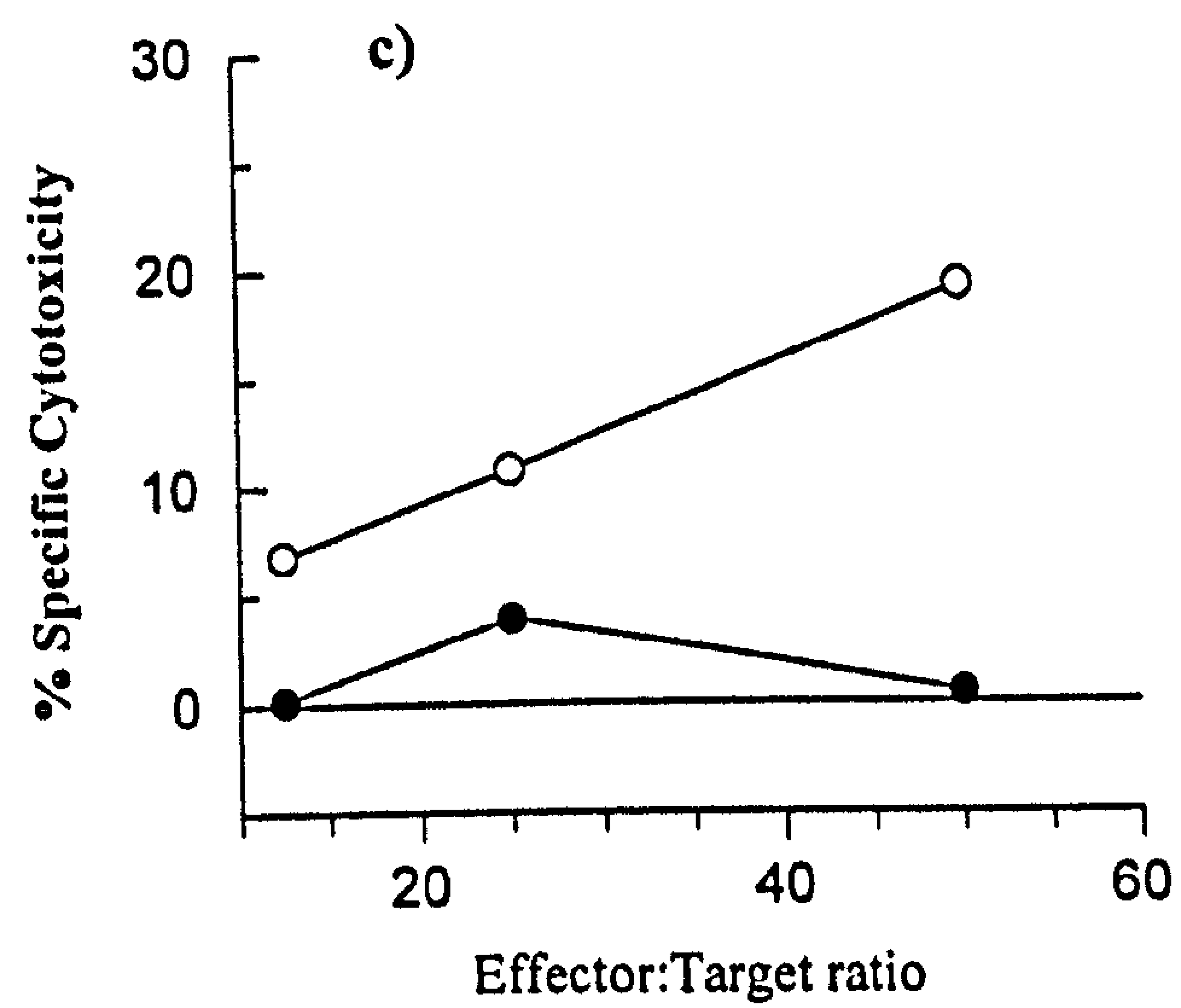
In another experiment (Fig. 3.9.) it was not possible to demonstrate CTL activity, above that seen when CTL were tested using autologous mock-infected target cells, in an 18 day old (Fig 3.9b) or a 4 month old foal (Fig 3.9.a). CTL activity was measurable from PBMC from adult mare (Fig. 3.9c). The data in Figures 3.8 and 3.9 suggest that foals become infected with either EHV-1 or EHV-4 before about 10 months of age. However, the possiblity exists that the CTL positive foal in Figure 3.8b had experienced more than one infection within 10 months, which makes it impossible to draw any conclusions about CTL induction after primary infection from these data.

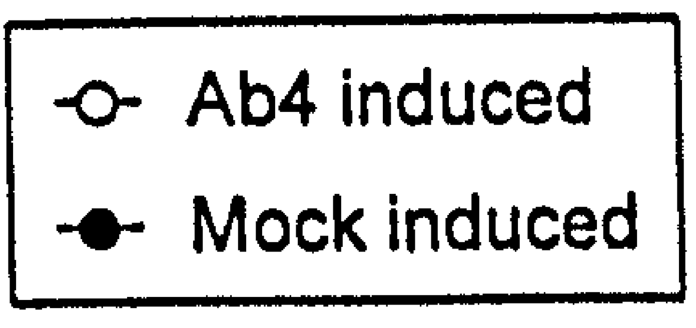
Figure 3.9. CTL activity in PBMC from young horses tested on autologous, EHV-1 infected target cells.

a) 4 month old foal.
b) 18 day old foal.
c) Adult mare.


a)
% Specific Cytotoxicity
30
20
10
0
10
20
30
40
50
60


b)
% Specific Cytotoxicity
30
20
10
0
10
20
30
40
50
60


c)
% Specific Cytotoxicity
30
20
10
0
20
40
60
Effector:Target ratio


Ab4 induced
Mock induced

The uncertainty about the number of infections experienced by young animals within the first year of life was overcome by measuring CTL activity in PBMC from EHV-1 challenge of SPF foals (Gibson *et. al.*, 1992a). The PBMC from this experiment were recovered from cryopreservation prior to CTL induction with EHV-1 strain Ab4. The results in Figure 3.10a and 3.10b show CTL activity from SPF foal #3. Although CTL activity from this animal was low after infection, CTL activity was seen at the 50:1 effector:target ratio. Figures 3.10c and 3.10d show CTL activity from SPF foal #4. CTL activity was seen after infection of this animal. This is formal proof that CTL activity can be induced after only one infection with EHV-1. The kinetics, duration and protective effect of this primary CTL response remain to be determined.

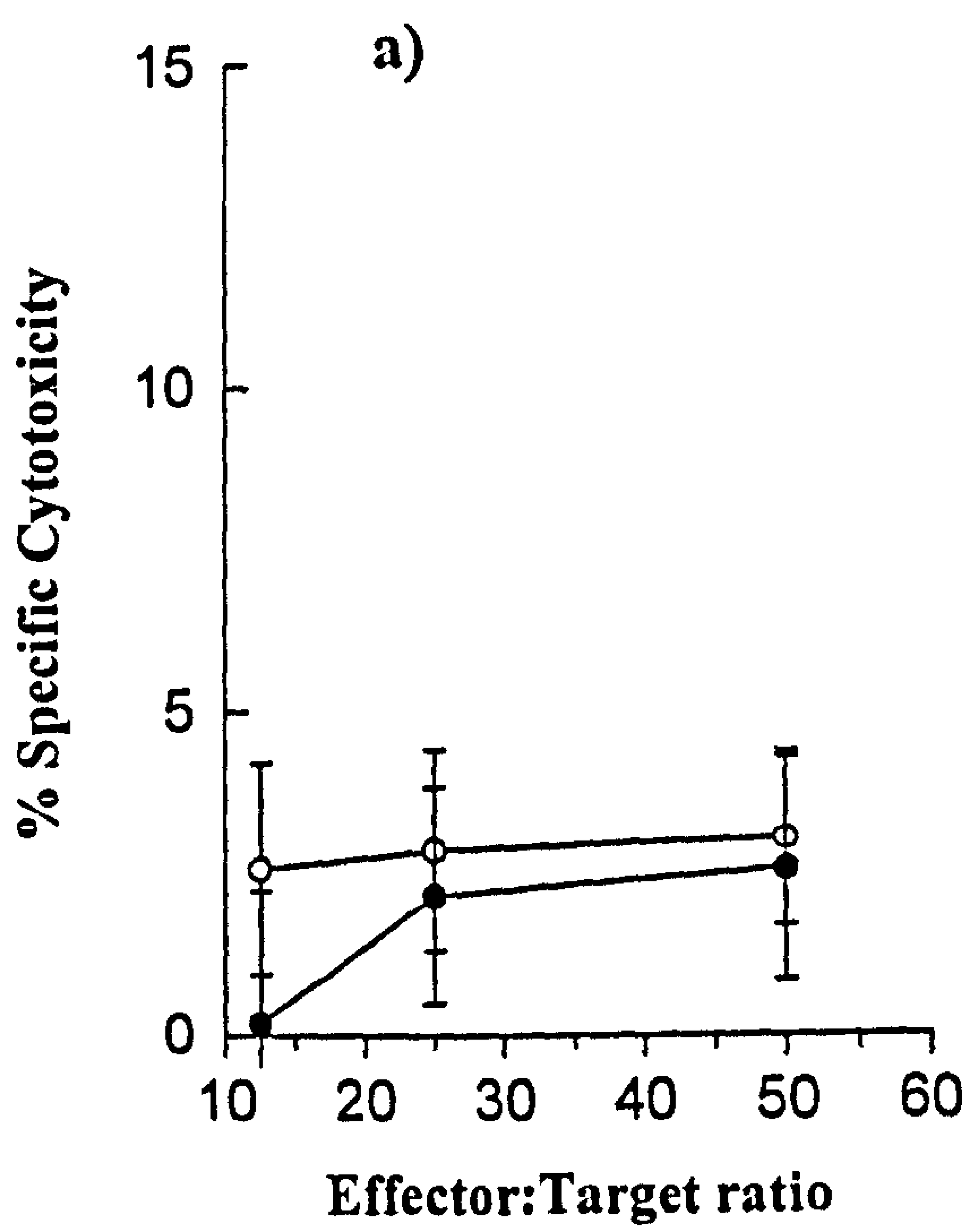
3.3.5. Cross-reactivity of induced CTL

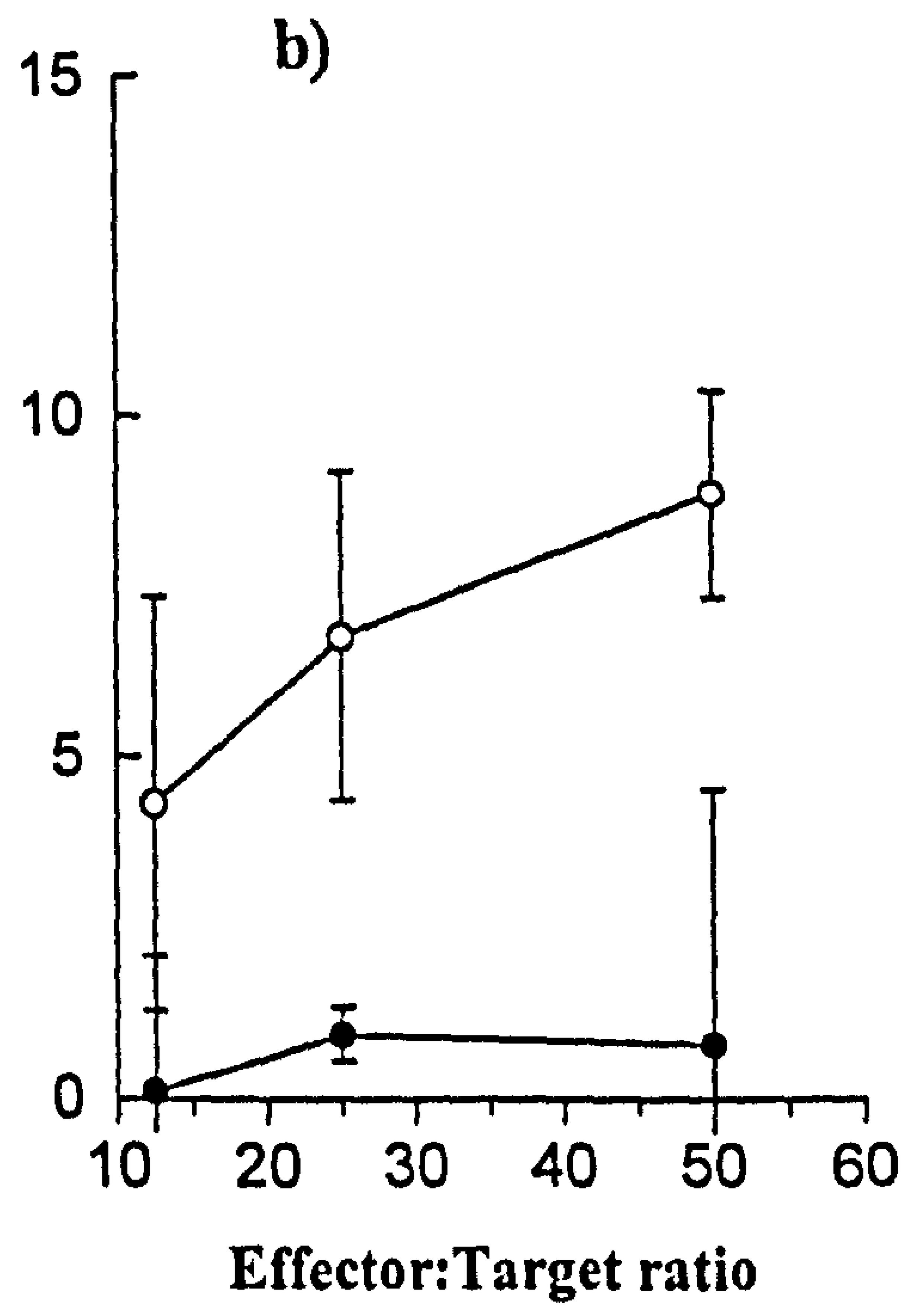
Consideration of the infection histories of young animals prompted an experiment to test whether CTL induced with various isolates of EHV-1 and EHV-4 were able to kill EHV-1 strain Ab4/13 infected target cells. As it was not possible to infect equine blast cells with EHV-4 to a high enough level to produce usable target cells, CTL were induced using this virus and assayed on autologous EHV-1 strain Ab4/13-infected target cells. This experiment also represented the first use of the cloned and sequenced EHV-1 virus Ab4/13 as the target antigen in virus-specific CTL assays. The results from this experiment can be seen in Figure 3.11.

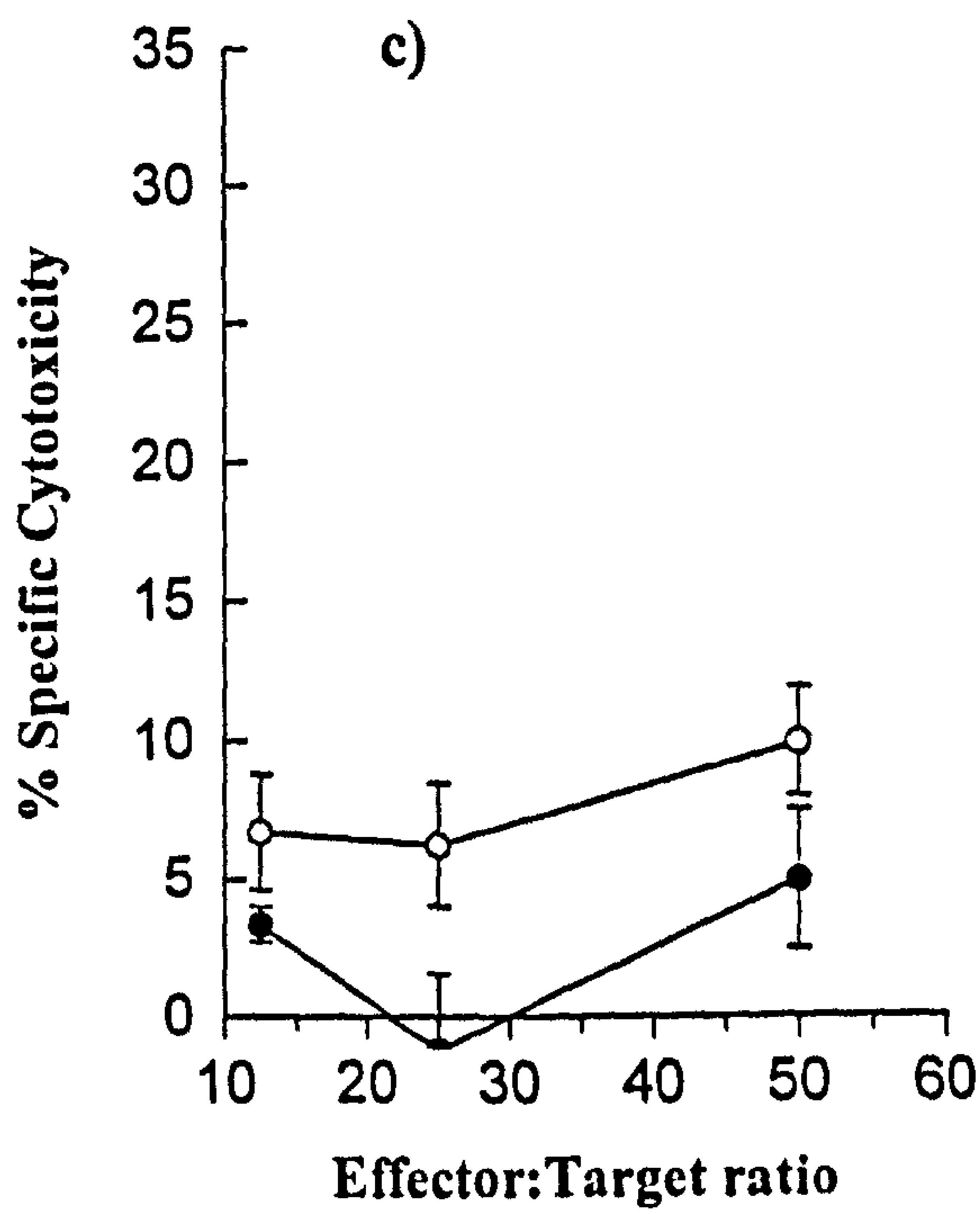
The results presented in Fig. 3.11 showed that different EHV-1 viruses as well as an

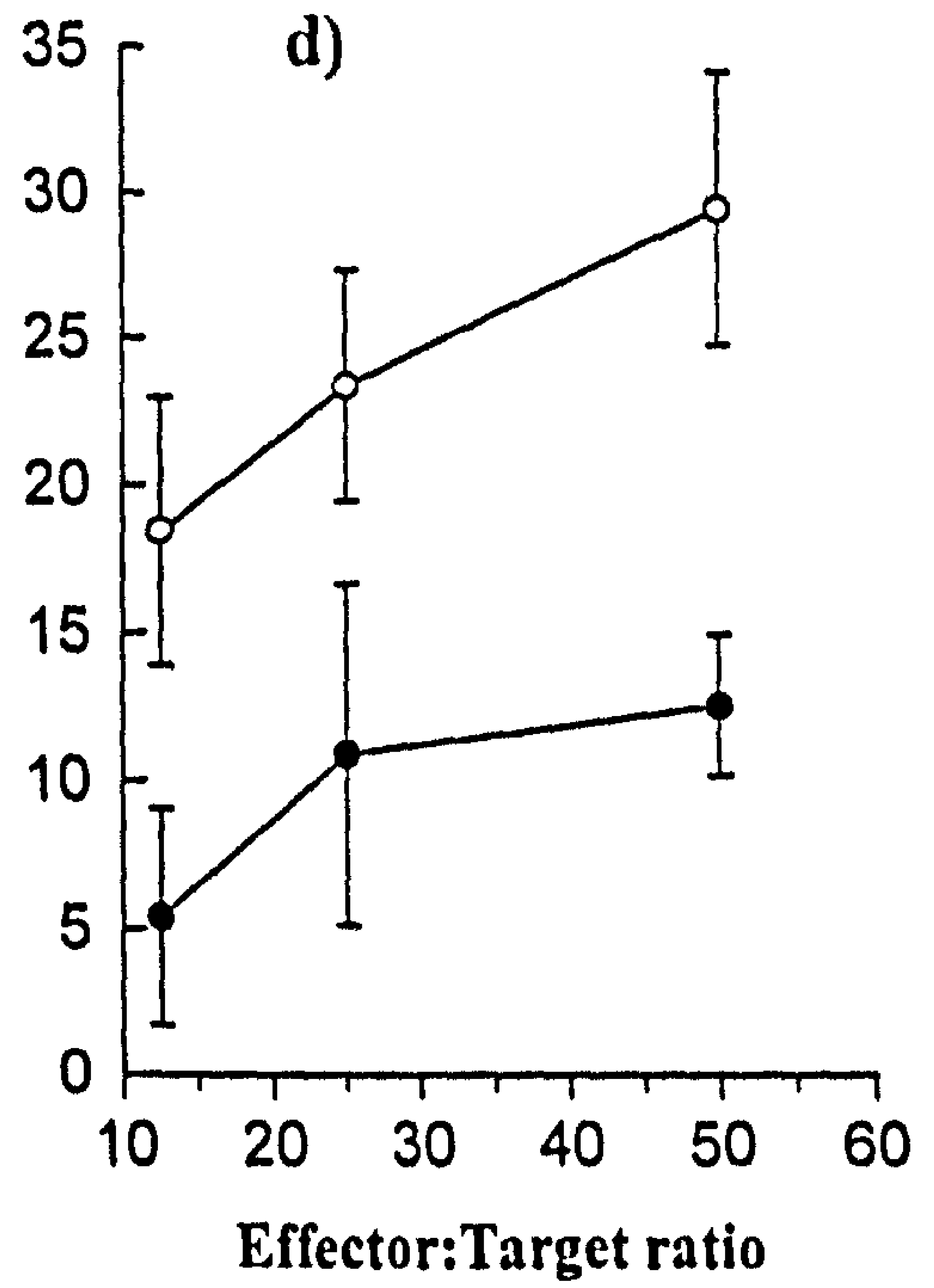
Figure 3.10. CTL activity in PBMC from 2 SPF (EHV free) foals before and after infection with EHV-1 strain Ab4/13.

a) Foal 3 before infection.
b) Foal 3 after infection.
c) Foal 4 before infection.
d) Foal 4 after infection.









Induction culture type

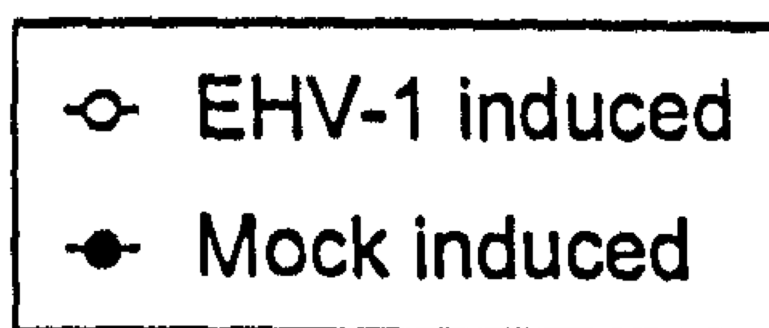
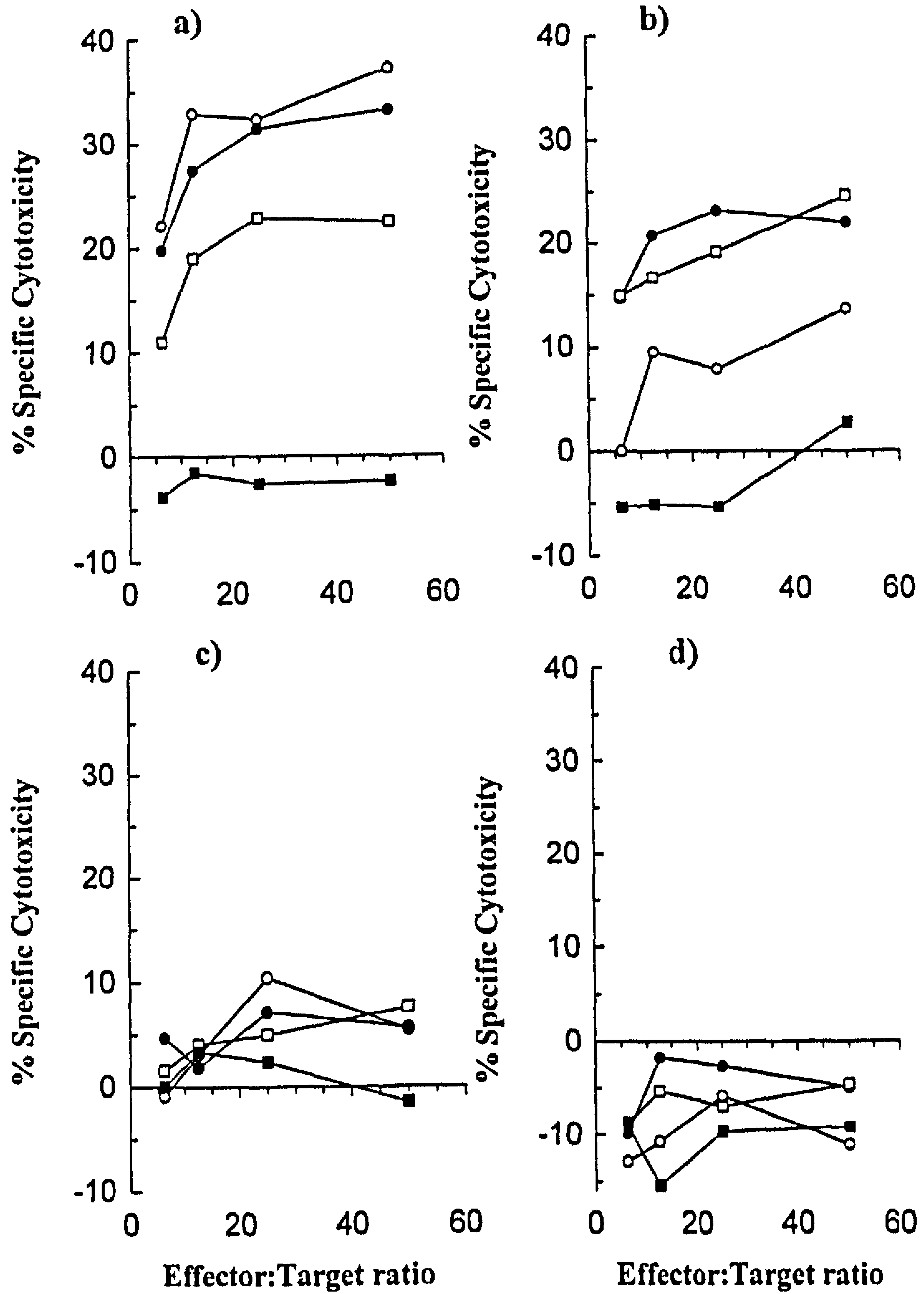


Figure 3.11. CTL induced using isolates of EHV-1 or EHV-4.

a) #302 effector cells on autologous, EHV-1 Ab4/13 infected targets.
b) #219 effector cells on autologous, EHV-1 Ab4/13 infected targets.
c) #302 effector cells on autologous, mock-infected targets.
d) #219 effector cells on autologous, mock-infected targets.


a)
b)
c)
d)
40
30
20
10
0
-10
0
20
40
60
% Specific Cytotoxicity
Effector:Target ratio
Induction culture type:
EHV-1 Ab4/9 induced
EHV-1 Ab4/13 induced
EHV-4 MD induced
Mock induced

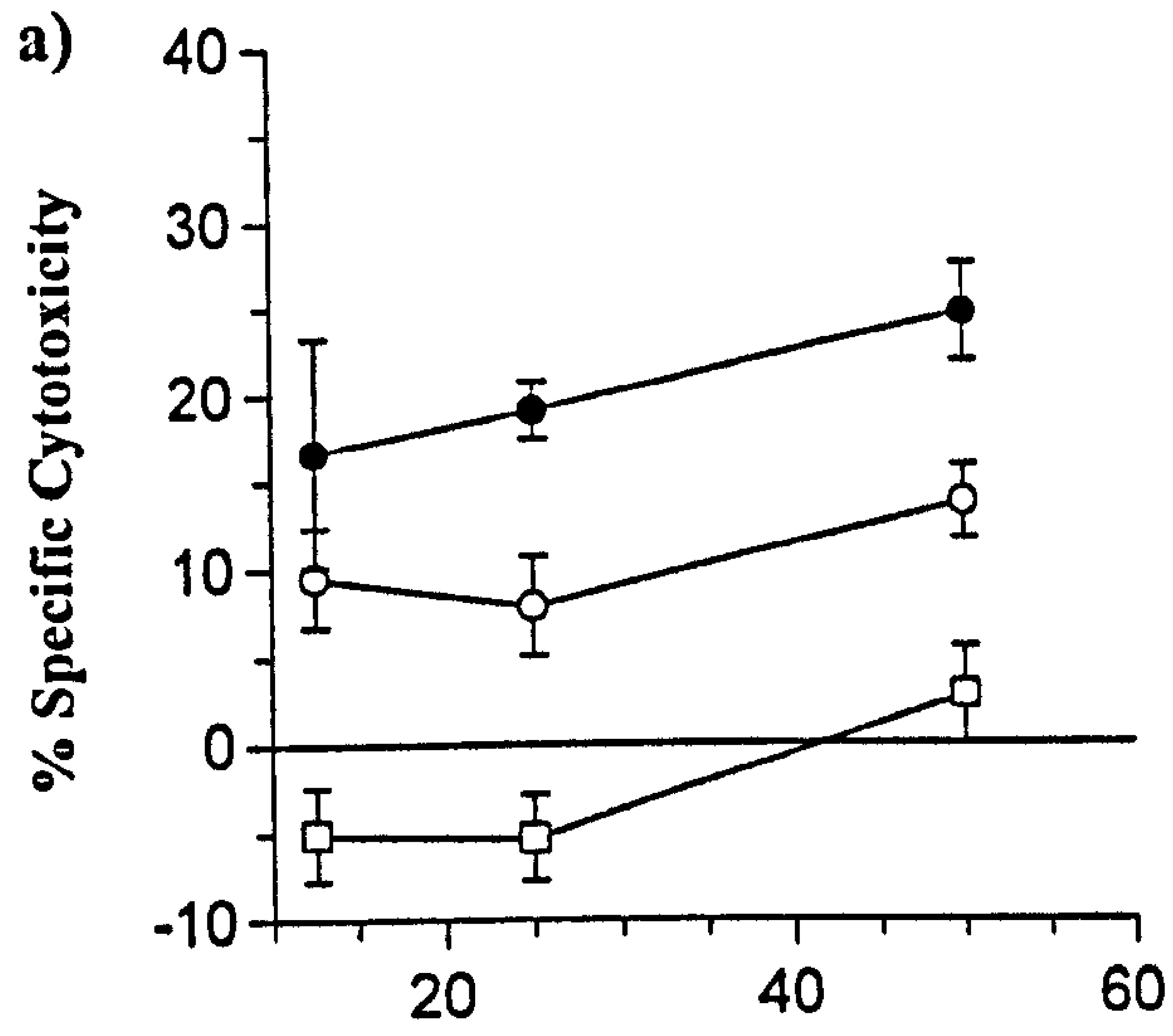
isolate of EHV-4 all induced CTL that killed targets infected with EHV-1 strain Ab4/13. In Fig. 3.11a (effector CTL from horse #302) equivalent levels of specific lysis against autologous EHV-1 Ab4/13 infected targets were measured using EHV-1 strains Ab4/9 and Ab4/13 as inducing viruses (between 20 and 35%), whilst significantly ($p<0.05$) reduced levels (between 10 and 20%) were seen when EHV-4 strain MD was used as the inducing agent at the same concentration. Killing of autologous mock-infected target cells was negligible (Fig. 3.11c). In Fig. 3.11b (effector CTL from horse #219) the pattern of reactivity of the induced CTL was very different. CTL from this animal induced with Ab4/13 and EHV-4 strain MD produced equivalent levels of specific lysis (around 20%) whilst CTL induced with Ab4/9 produced significantly ($p<0.05$, except at 12.5:1 E:T ratio which was not significant) lower values (approximately 0-10%). Again, killing of autologous mock-infected target cells was negligible (Fig. 3.11d).

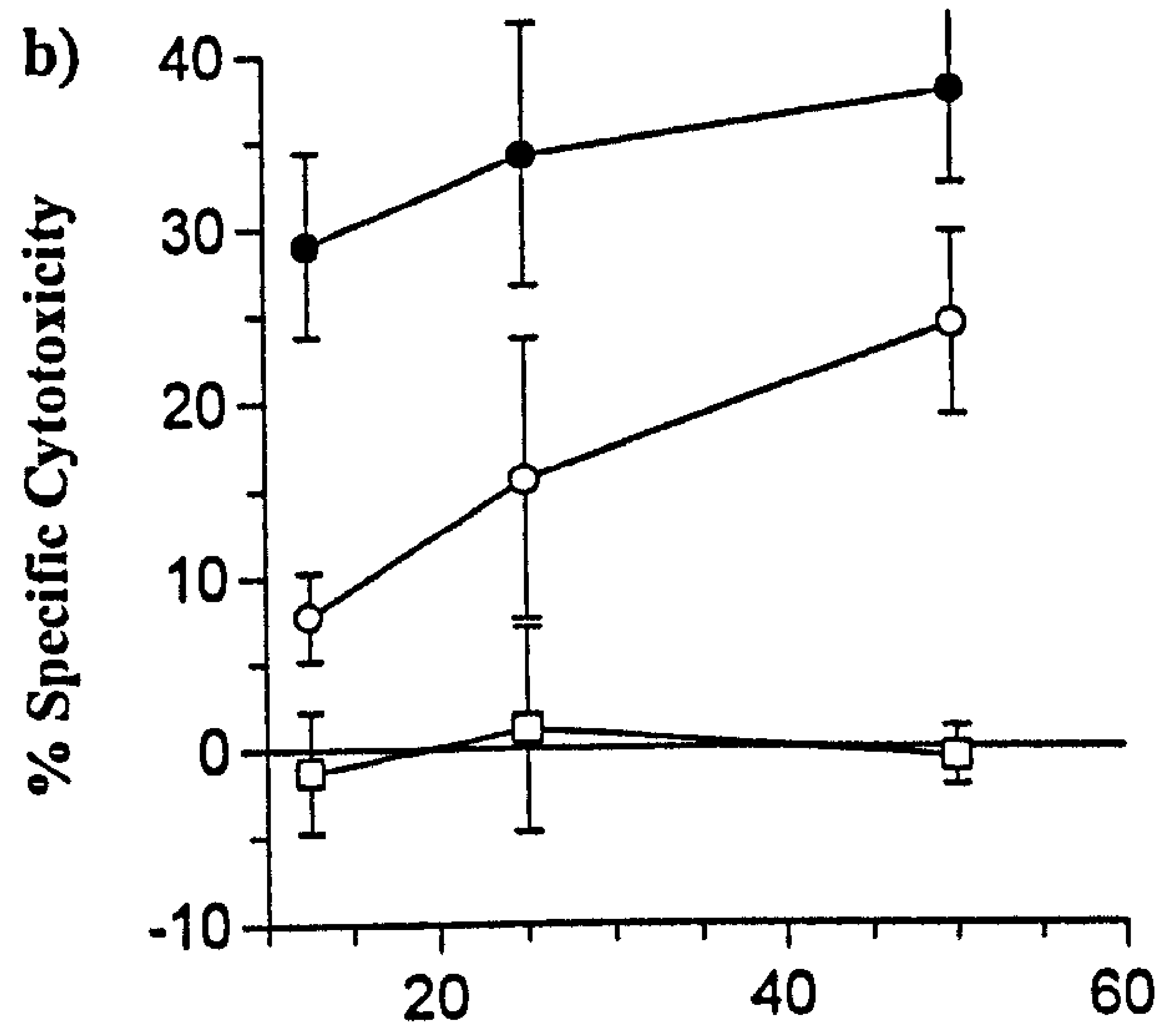
The stability of the CTL induction pattern induced by the different viruses was studied by comparing levels of CTL activity in PBMC from a single immune animal (horse #219) on three separate occasions. Figure 3.12. shows that, despite differences in the absolute levels of % specific cytotoxicity, the pattern of CTL reactivity seen 68 days after infection was maintained until 238 days after infection.

These results show that different levels of CTL activity can be induced from the same initial population of PBMC using different viruses. The CTL induced with EHV-4 and assayed on EHV-1 infected targets will be referred to as cross-reactive CTL. The pattern

Figure 3.12. CTL induced with EHV-1, EHV-4 or mock antigen in PBMC from mare #219 on 3 occasions after EHV-1 infection.

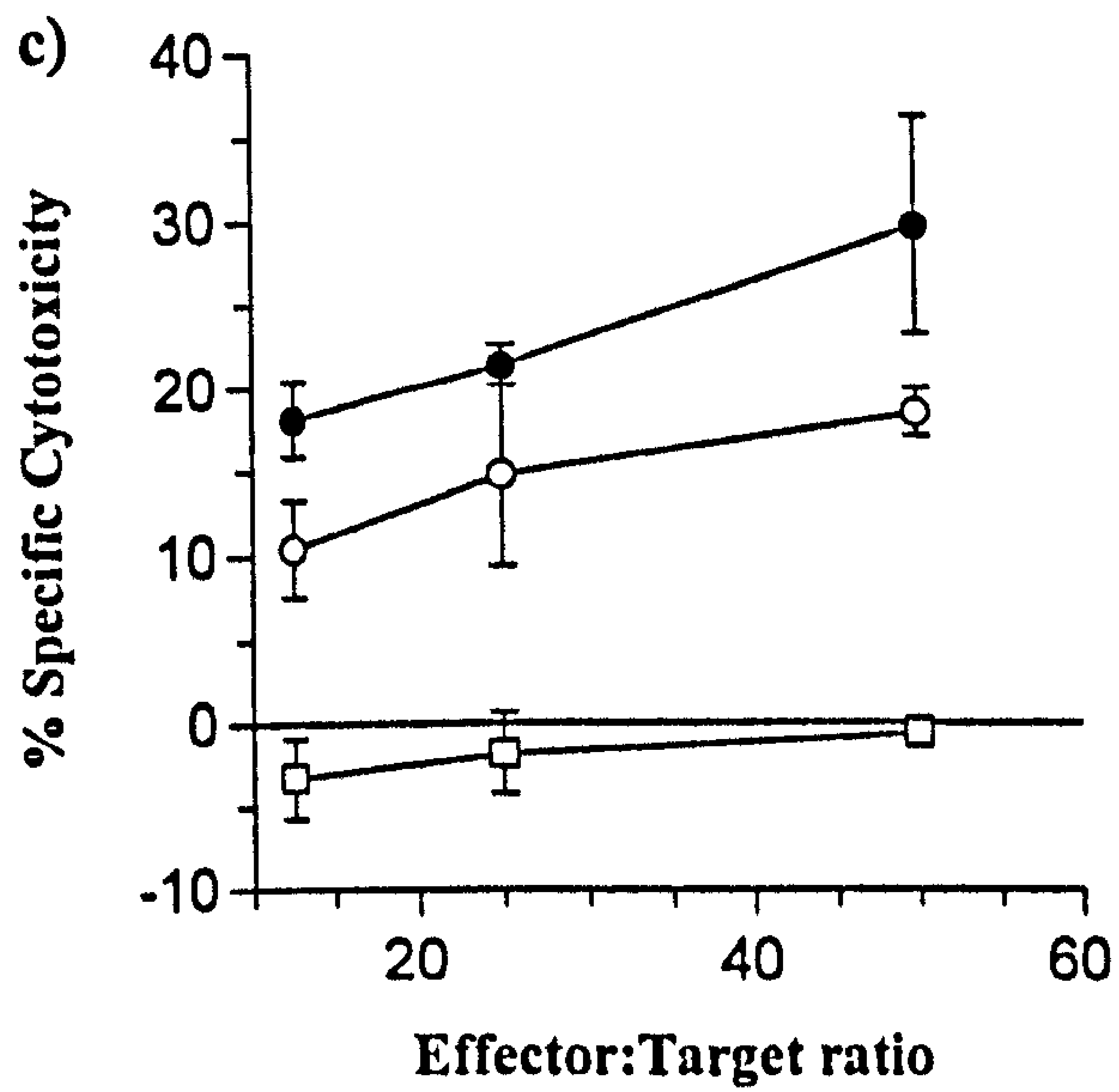
a) 68 days after infection.
b) 203 days after infection.
c) 238 days after infection.





Inducing Antigen:

- Ab4
- MD
- No Ag



of CTL reactivity induced was maintained over a long period and may be a function of the infection history of the individual horses or it may be related to MHC type.

3.3.6. The effect of rhIL2 on EHV-1 specific CTL induction

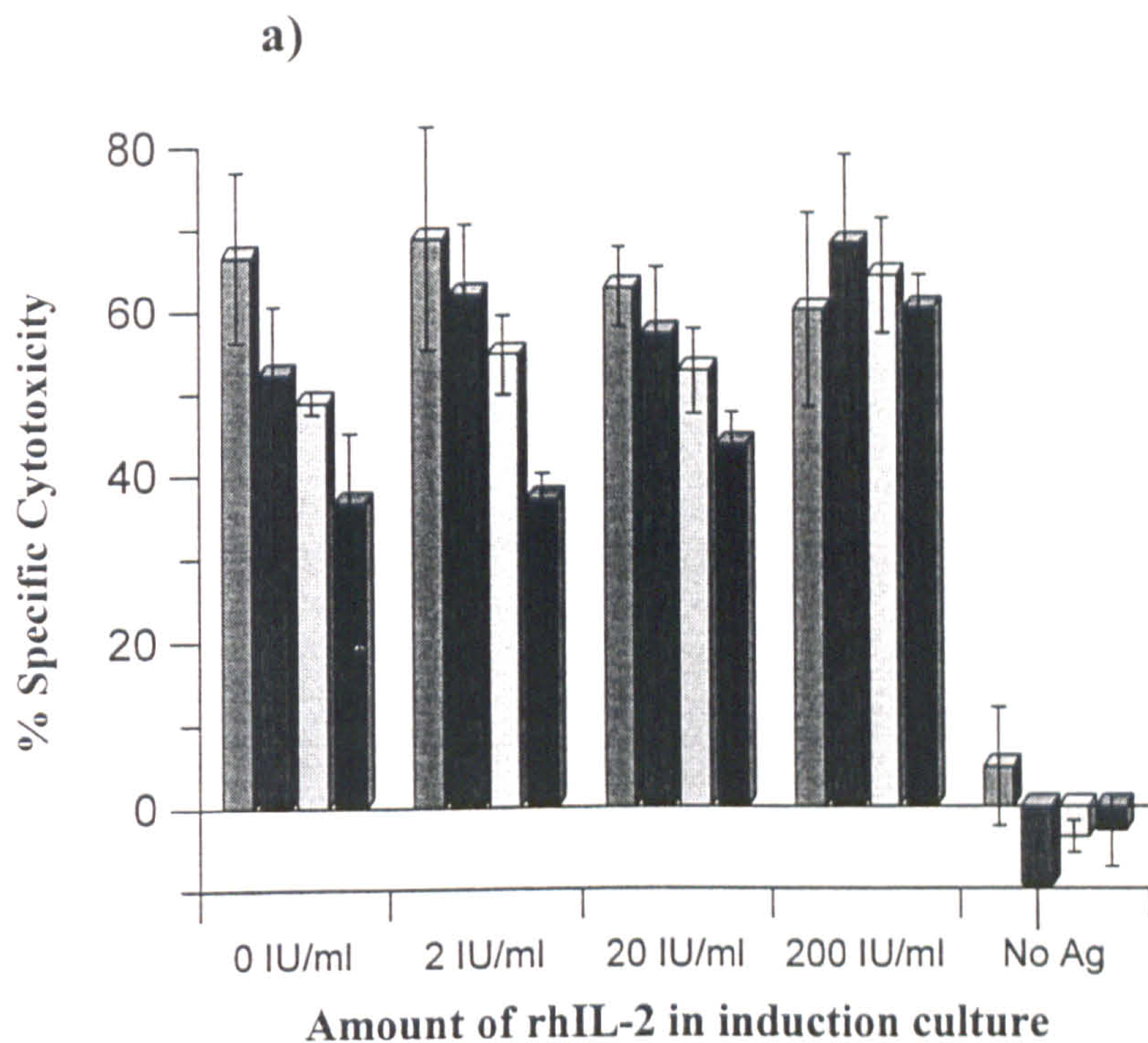
The autocrine T cell growth factor Interleukin 2 (IL2) plays a central role in the induction of CTL specific for many antigens in many species (Smith, 1988). In order to assess the effect of IL2 on the induction of equine EHV-1 specific CTL induction cultures were set up containing different amounts of rhIL2.

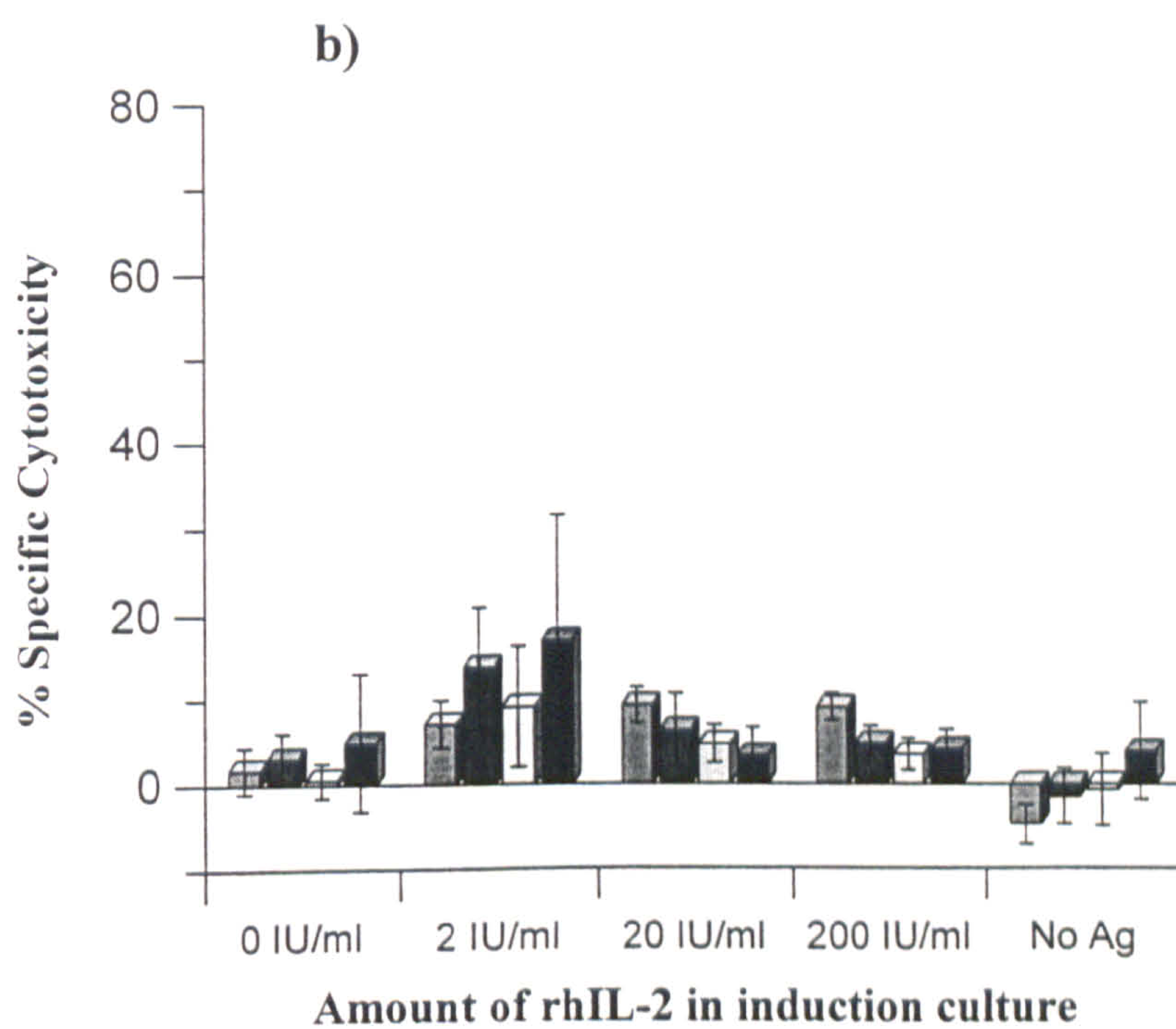
The results in Figure 3.13 show that inclusion of rhIL2 in the CTL induction culture had little effect on the maximum levels of lysis of autologous or allogeneic target cells. However, the levels of killing obtained at low effector:target ratios were enhanced. This may reflect a higher frequency of effector CTL induced in these cultures due to the rhIL2. The inclusion of high levels of rhIL2 in the induction culture had no effect on the genetic restriction of the resultant CTL. Figure 3.14 shows results from the same experiment using #219 effector cells. Again, at 200 $IUml^{-1}$ rhIL2 killing obtained at low effector:target ratios were enhanced. Using PBMC from #219 and 2 $IUml^{-1}$ rhIL2 the CTL activity became genetically non-restricted at 50:1 effector:target ratio only. This result was difficult to explain and may be an experimental artifact.

These results show that the inclusion of rhIL2 in the induction cultures had little obvious effect on either the levels of CTL induced or their genetic restriction. However, the

Figure 3.13. Effect of rhIL-2 on the induction of EHV-1 specific CTL.

a) Mare #302 effector cells on autologous EHV-1 infected targets.
b) Mare #302 effector cells on allogeneic EHV-1 infected targets.





Effector:Target ratio:

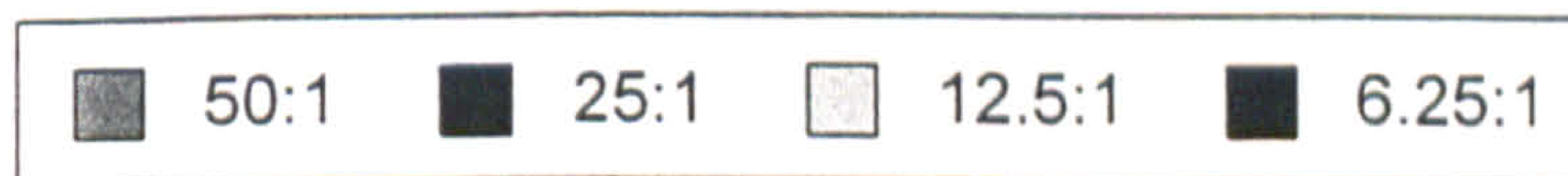
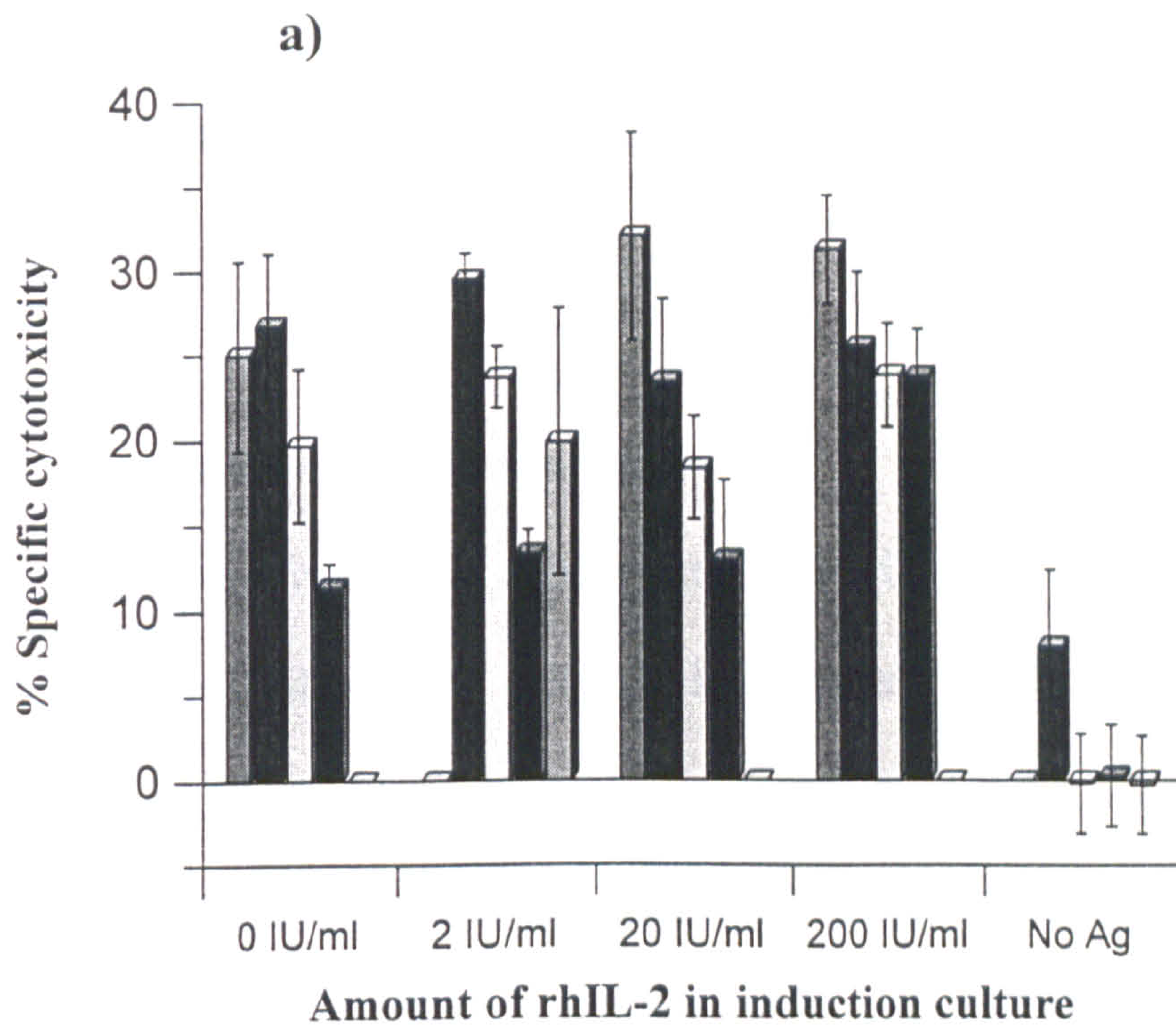
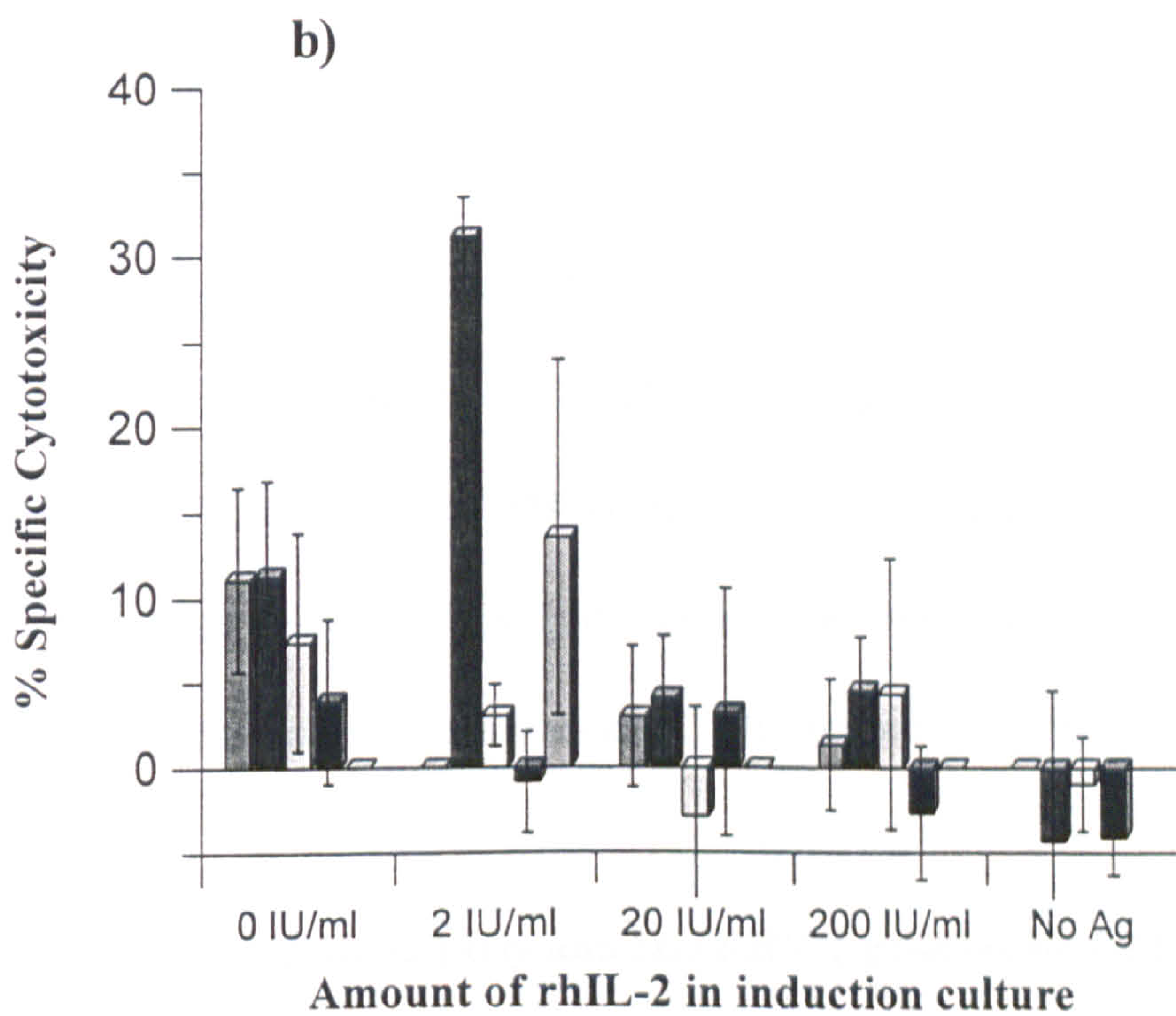


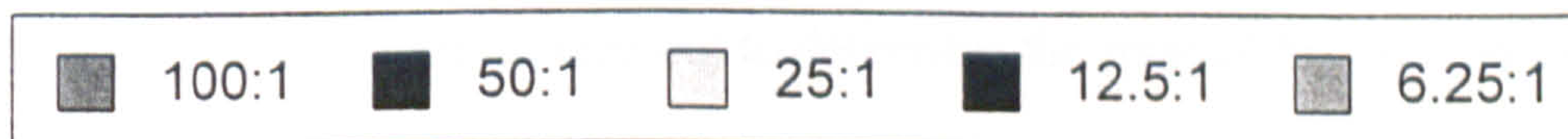
Figure 3.14. Effect of rhIL-2 on the induction of EHV-1 specific CTL.

a) Mare #219 effector cells on autologous EHV-1 infected targets.
b) Mare #219 effector cells on allogeneic EHV-1 infected targets.





Effector:Target ratio:



increase in killing at low effector:target ratios when high levels of rhIL2 were used may reflect an increase in the efficiency of maturation of precursor CTL to effector CTL under these conditions.

3.3.7. CTL activity in equine T cell sub-populations

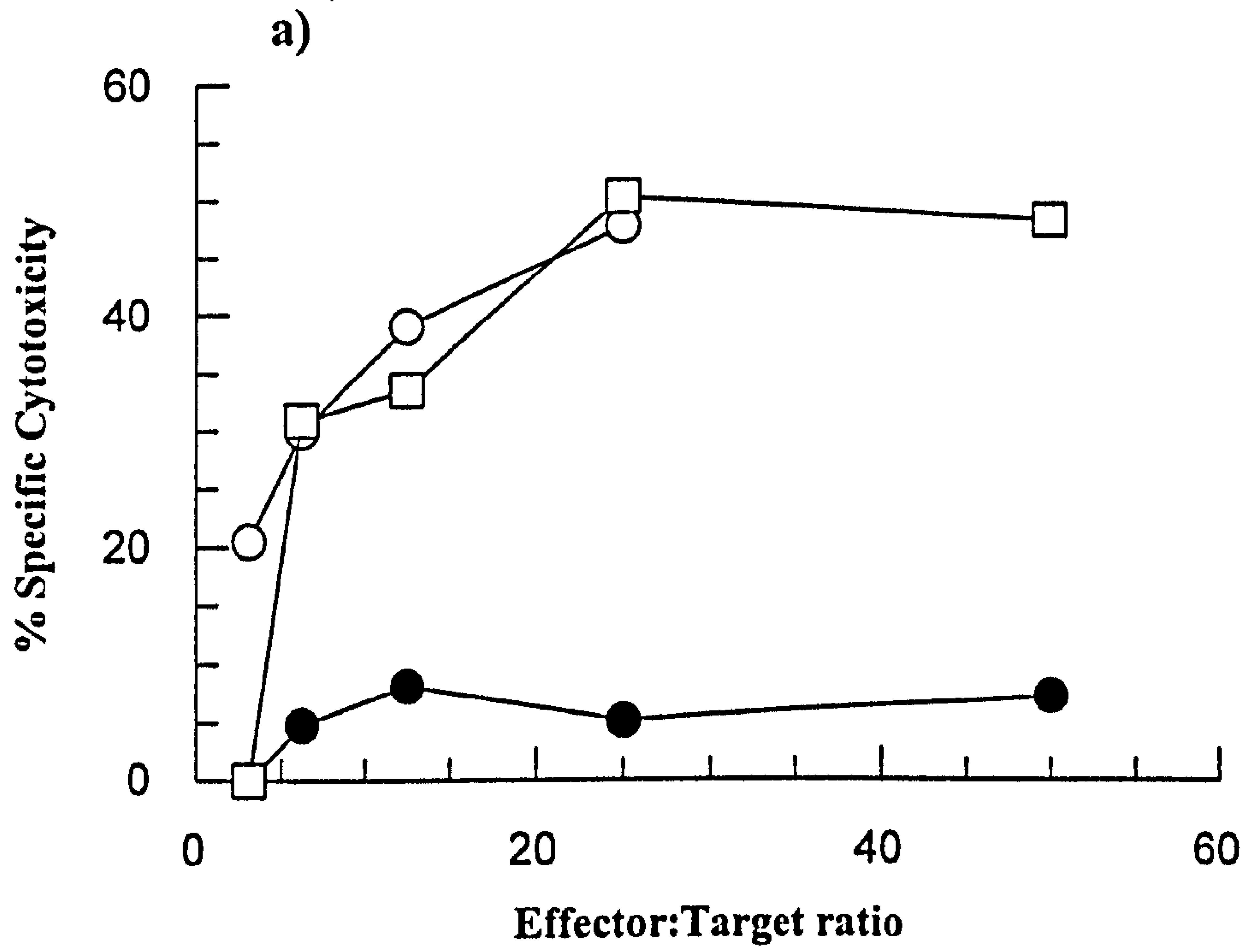
Figure 3.15 shows the results from an experiment in which CTL induced with live EHV-1 were depleted of either $CD4^+$ or $CD8^+$ cells and then used as effectors in a standard EHV-1 specific CTL assay. The resultant CTL were assayed on three target cell types, namely, autologous infected, allogeneic infected and autologous mock-infected. Very little killing was observed when any of the effector populations were assayed on allogeneic infected or autologous mock-infected target cells. Depletion of $CD4^+$ cells from the effector population had no significant effect on killing of autologous infected target cells when compared with effector cells treated with the anti-equine thymocyte monoclonal antibody which has been shown not to bind to equine PBMC and therefore was used as a negative control. However, CTL depleted of $CD8^+$ cells did not kill autologous infected target cells above background levels. This is confirmation that EHV-1 specific CTL activity induced using live virus resides in the sub-set of cells expressing CD8 on the cell surface.

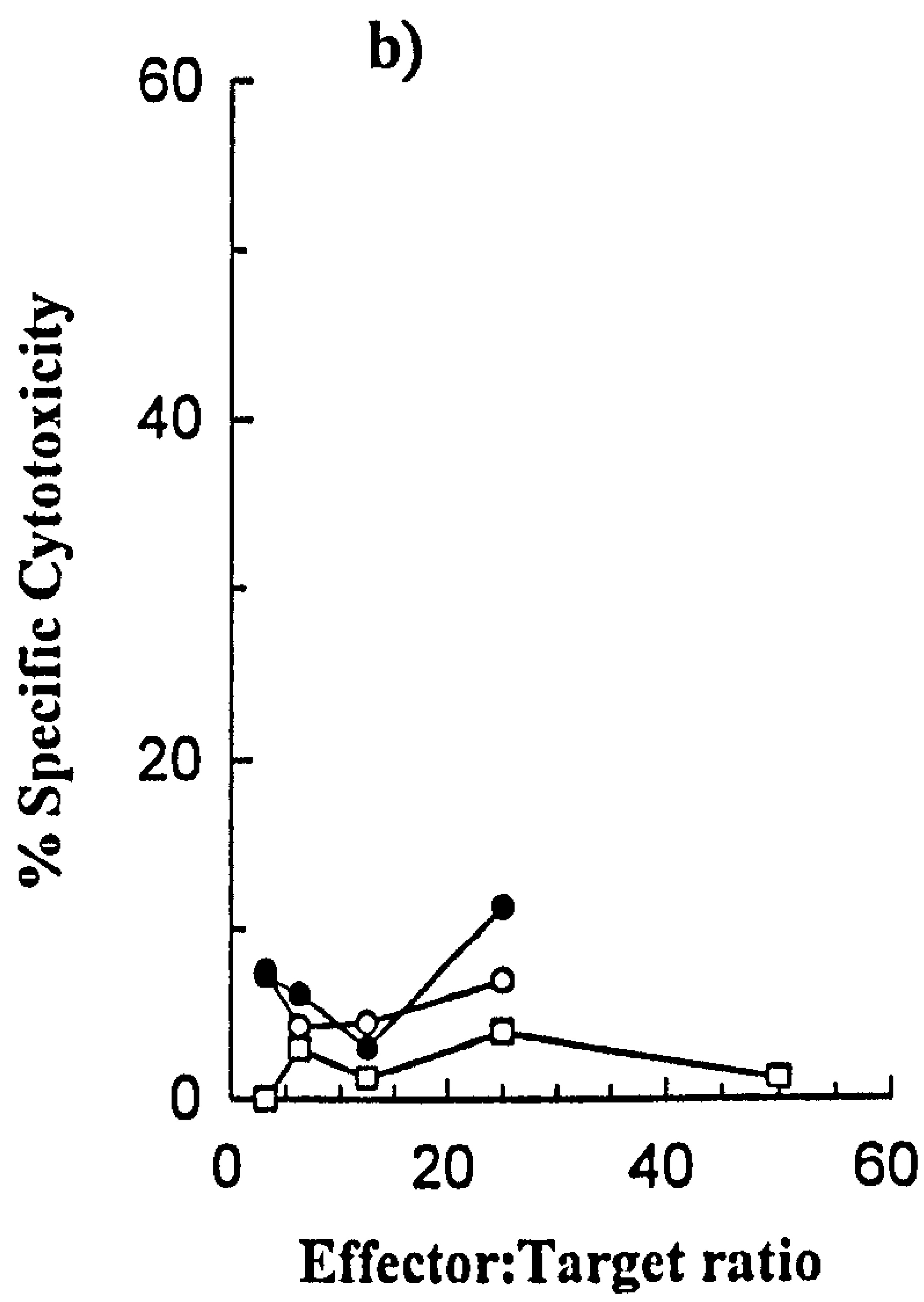
3.3.8. Recognition of immediate early and late EHV-1 proteins by CTL

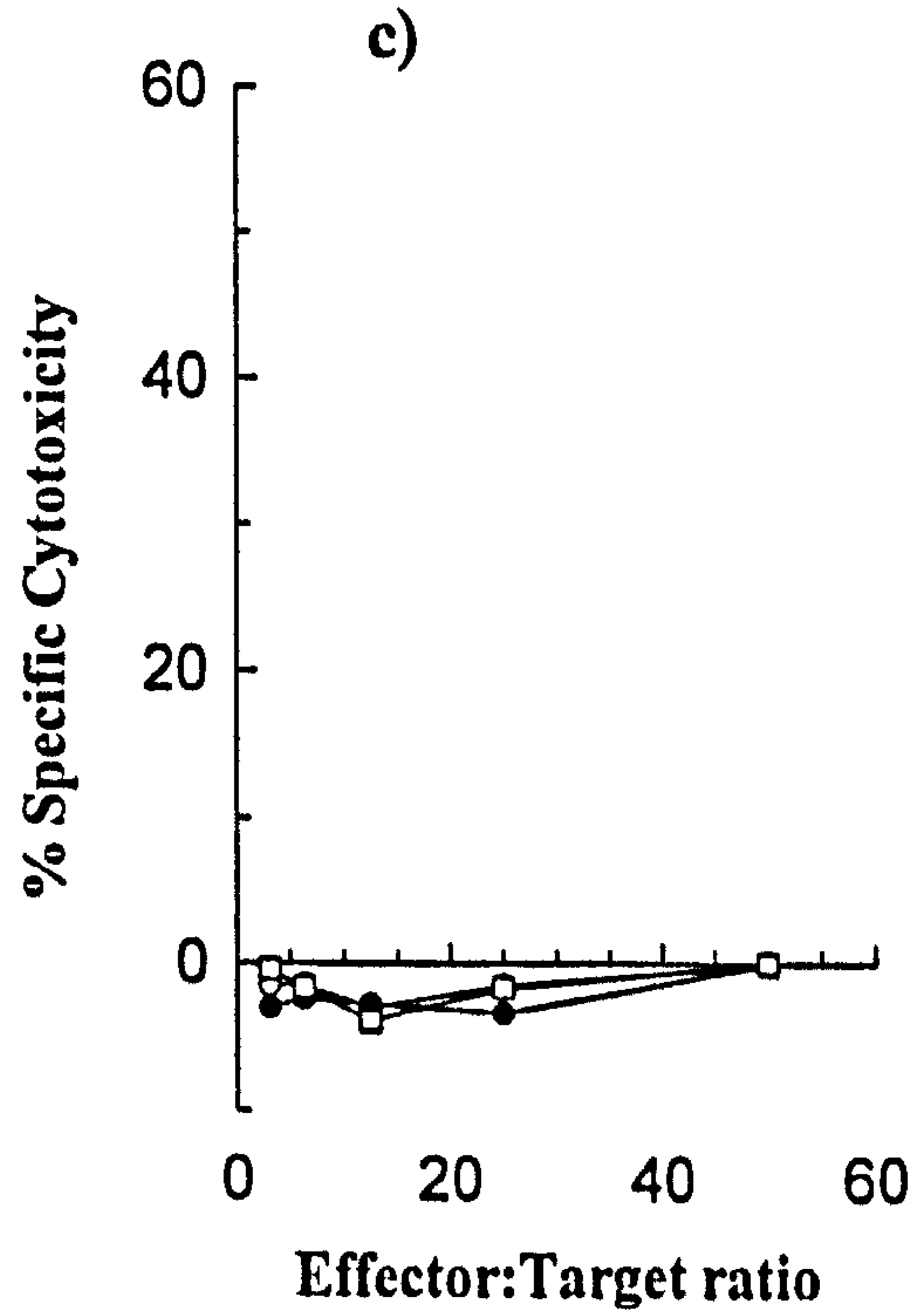
As a first step towards the delineation of the specific antigens recognised by EHV-1 specific CTL, experiments were carried out to determine the stage of the viral replication

Figure 3.15. ***In vitro*** **depletion of T cell sub-sets from EHV-1 specific effector cells.**

a) Autologous EHV-1 strain Ab4/9 infected targets.
b) Allogeneic EHV-1 strain Ab4/9 infected targets.
c) Autologous Mock infected targets.







Effector cells depleted of:

cycle at which CTL target antigens are expressed. EHV-1 specific CTL were induced with live virus and then assayed for CTL activity against autologous infected and mock-infected cells which had been treated with cycloheximide and actinomycin D in order to restrict expression of viral proteins to the immediate early (IE) proteins only. The results are presented in Figure 3.16.

Figures 3.16a and 3.16b show that assay of CTL on autologous, infected, untreated target cells resulted in high levels of specific killing whilst assay of the same CTL on autologous, mock-infected, untreated target cells resulted in no specific killing.

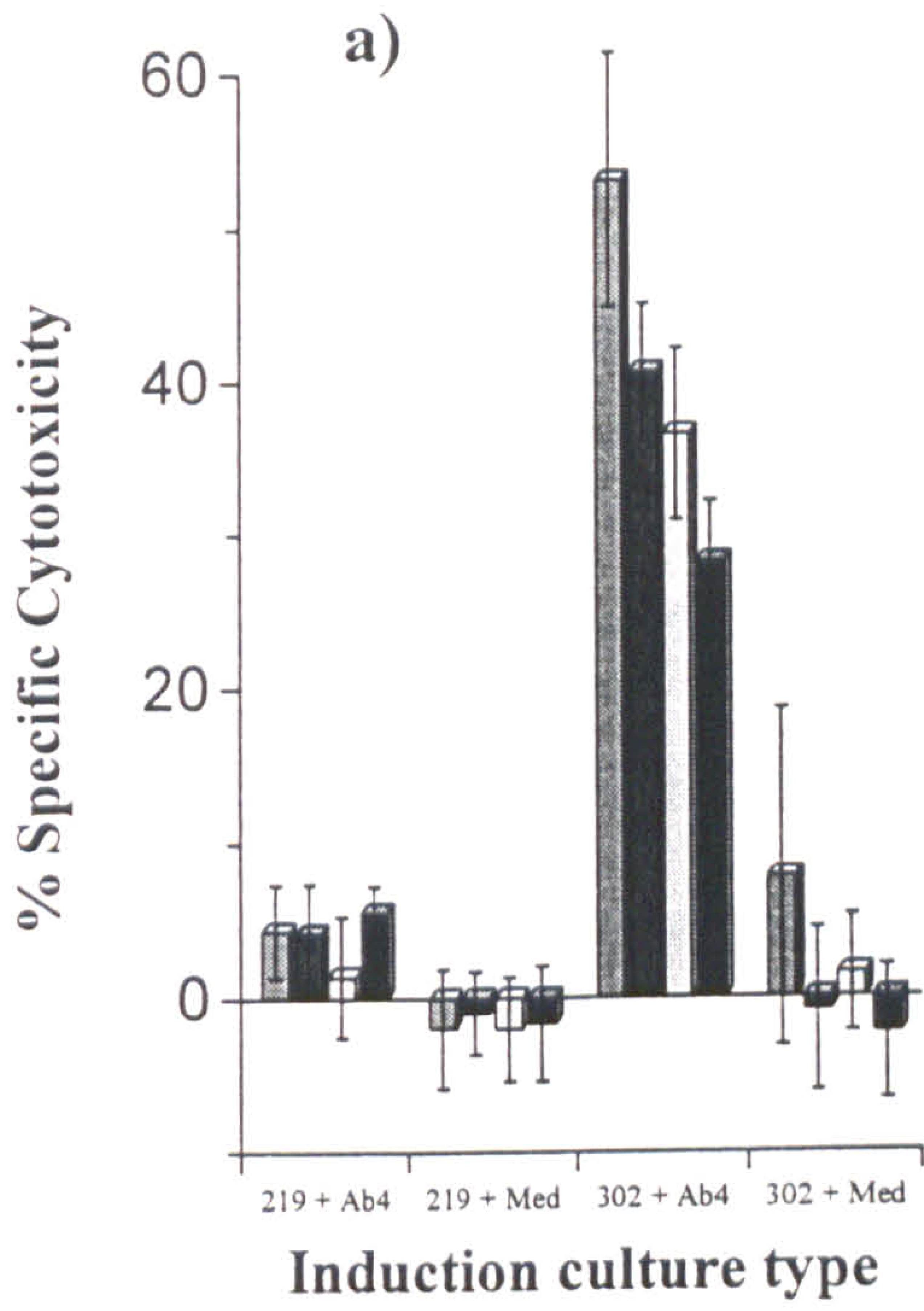
Assay of the same CTL on autologous, infected target cells treated with cycloheximide and actinomycin D (Fig. 3.16c) resulted in reduced levels of specific cytotoxicity. However, the % specific lysis of the cycloheximide treated infected targets was significantly higher ($p<0.01$ at E:T ratios of 50:1 and 25:1 only) than that of cycloheximide treated mock-infected targets (Fig. 3.16d). This preliminary result shows that IE gene products may have been recognised by effector CTL induced with live EHV-1 although further work is necessary in order to confirm that only IE mRNA was produced in the cycloheximide/Actinomycin D treated target cells.

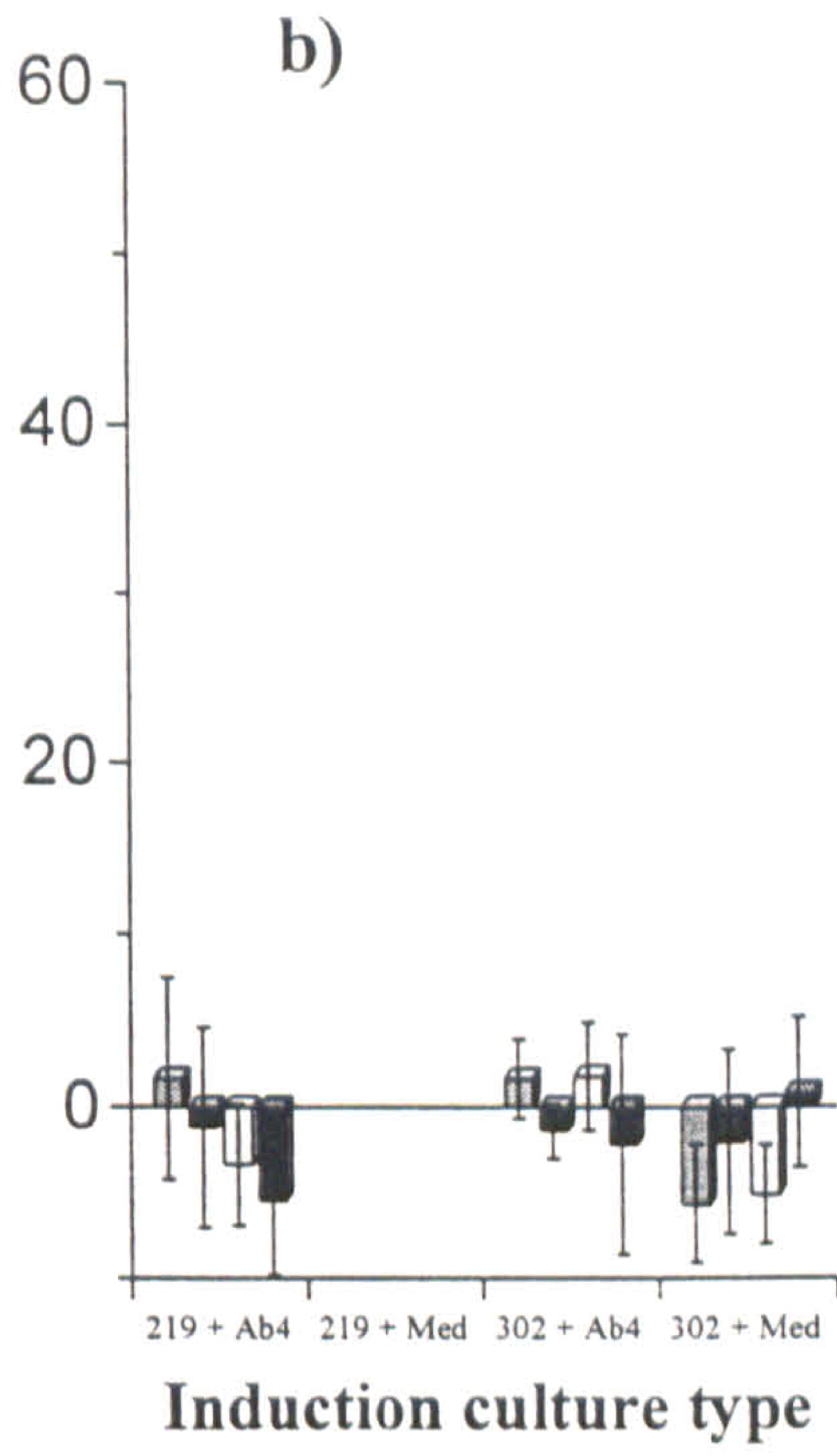
3.4 Discussion

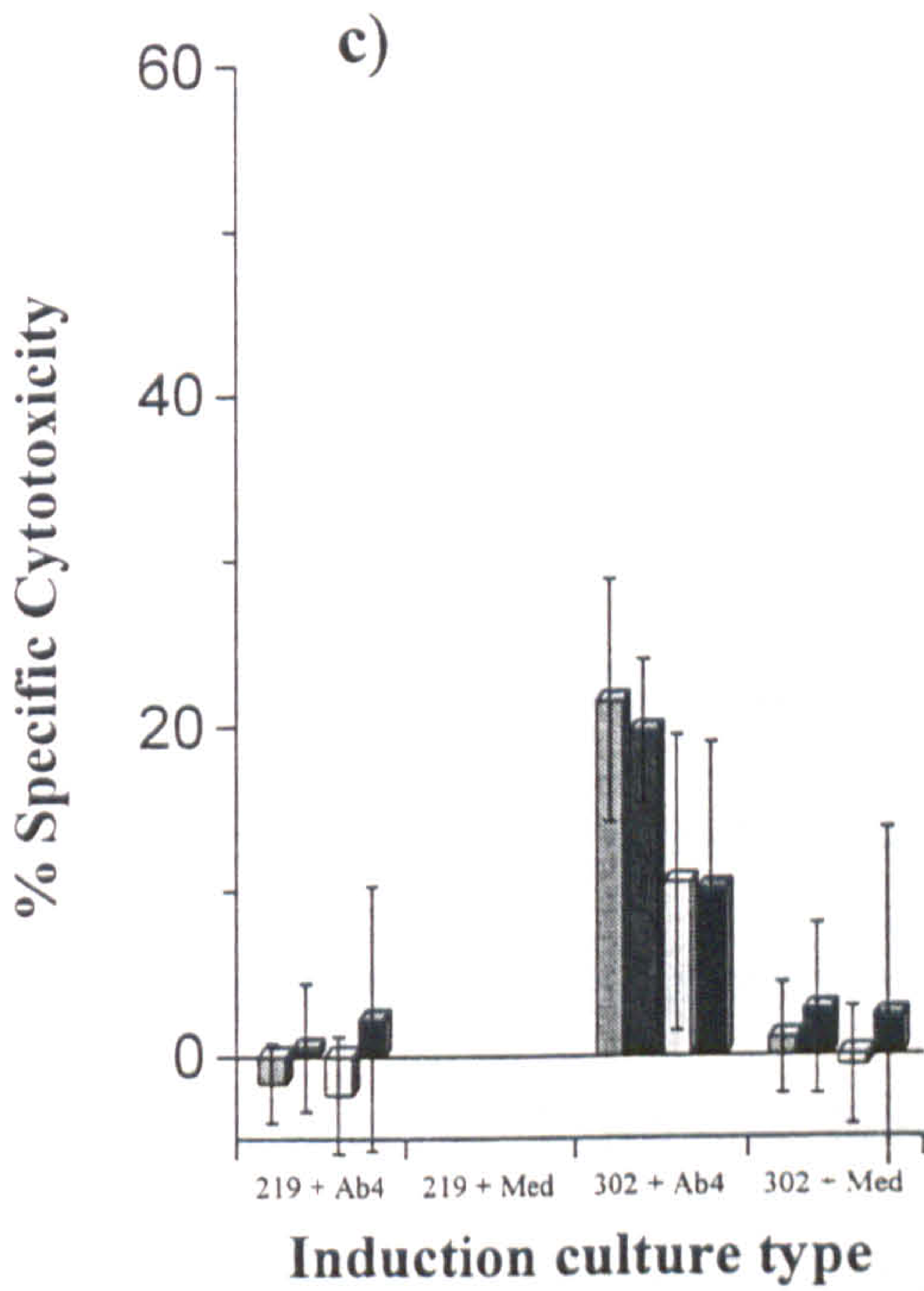
In this chapter results were presented from experiments designed to characterise the

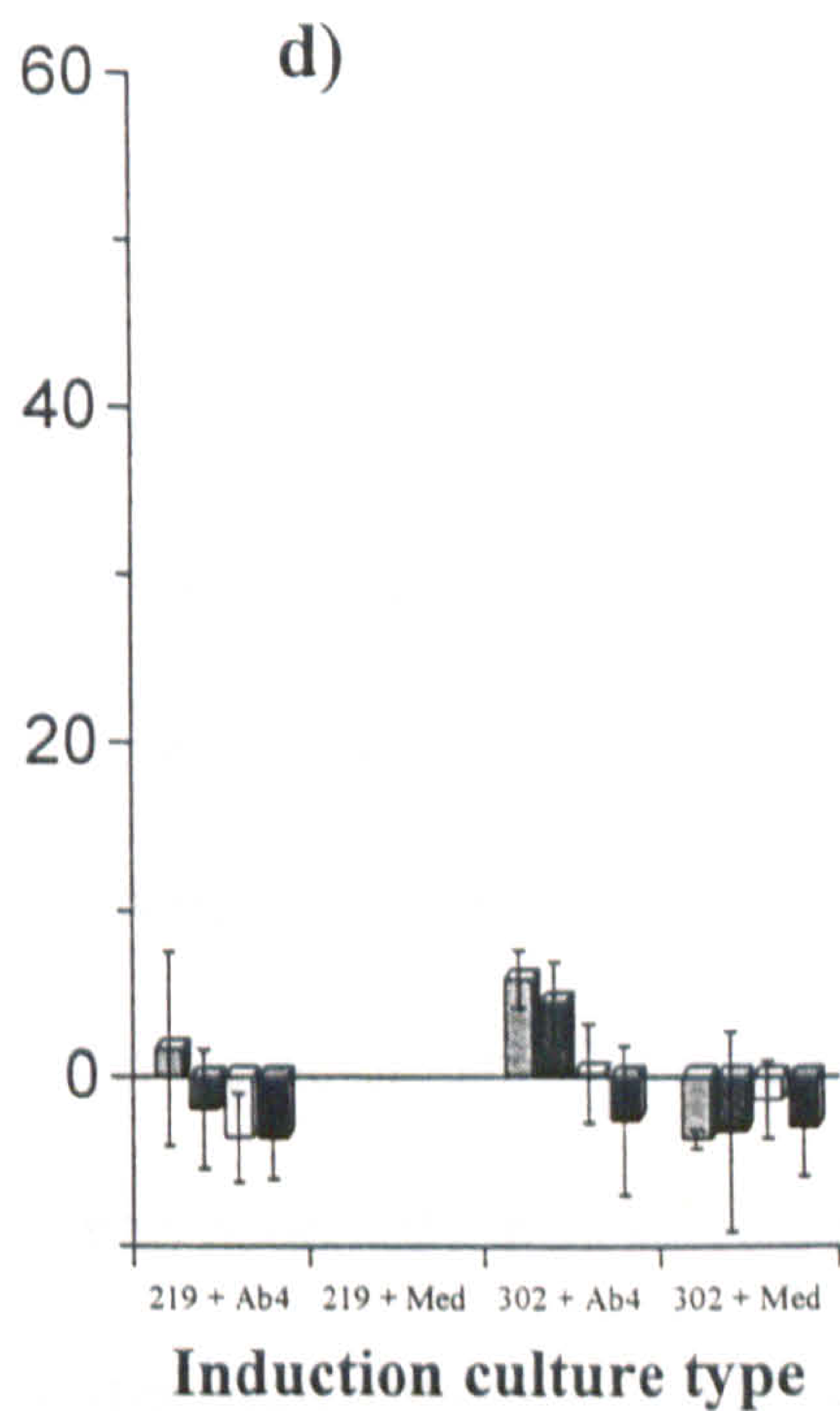
Figure 3.16. Recognition of EHV-1 IE protein antigens by EHV-1 specific CTL induced in PBMC from mare # 302.

a) Autologous, EHV-1 infected targets.
b) Autologous, mock infected targets.
c) Autologous, EHV-1 infected, Cycloheximide treated targets.
d) Autologous, mock infected, Cycloheximide treated targets.

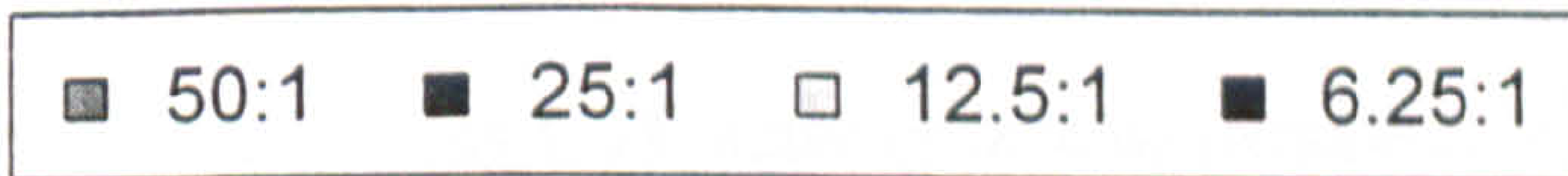








Effector:target ratio



EHV-1 specific CTL assay developed by Allen *et. al.* (1995) with EHV-1 strain A183. The assay was optimised for use under our laboratory conditions in the light of our previous experience in handling cytolytic cells and with the eventual aim of using the technique for the measurement of CTL activity under limiting dilution conditions. The assay was also used to extend the results of Allen *et. al.* (1995) to include studies to identify which class of viral proteins were recognised by polyclonal CTL. As there are few other reports of assays measuring virus-specific CTL activity from horses it is not possible to discuss the results in the light of direct current knowledge. However, there is a vast body of work on herpesvirus specific CTL in other mammalian species and discussions of my work will take this into account.

In general, *in vitro* incubation in the presence of specific antigens is required in order to differentiate CTL precursors (CTLp) into fully active cytolytic effector CTL (Lawman *et. al.*, 1980b, Lawman *et. al.*, 1981, Hannant & Mumford 1989). Exceptions to this rule are CTL from humans infected with cytomegalovirus (Quinnan *et. al.*, 1982), CTL from a proportion of humans infected with HIV (Rowland-Jones *et. al.*, 1995) and CTL from equine infectious anaemia virus-infected horses (McGuire *et. al.*, 1994). The first reports of CTL specific for herpes simplex virus (HSV) in the mouse model of human disease (Pfizenmaier *et. al.*, 1977) required that draining lymph node cells from sub-cutaneous injection of virus were cultured for 2-3 days before specific cytotoxicity could be demonstrated. One explanation for this need for *in vitro* incubation was that suppressor mechanisms were at work regulating CTL activity *in vivo* (Rouse & Horohov, 1984).

This appeared to be supported by experiments which showed that pre-treatment of mice with cyclophosphamide, a suppressor T cell (Ts_I) inactivator (Asherson *et. al.*, 1980), enhanced both allospecific (Rollinghoff *et. al.*, 1977) and HSV-specific (Lawman *et. al.*, 1980b) CTL. It is clear from the results presented in 3.3.1. that equine EHV-1 specific CTLp require a period of *in vitro* incubation in the presence of viral antigens before antigen specific, genetically restricted CTL activity becomes measurable. There is evidence (see Fig. 3.1) that CTL activity generated early after initiation of *in vitro* induction culture was not genetically restricted. This activity, which was lost by day 4 of the culture, may have been due to NK or LAK cells generated as a result of the initial burst of endogenous lymphokine production. However, EHV-1 specific CTL induced for 6 days and which killed autologous, infected target cells, did not kill the equine lymphosarcoma cell line EqT8888, which is sensitive to LAK cell killing (data not shown). The increase in the amount of allo-specific killing with increased time in culture seen with CTL from horse #302 (Fig. 3.2) is difficult to explain. The use of EqT8888 as a target for these genetically non-restricted CTL would determine whether the activity is LAK cell or NK cell mediated.

In the induction cultures used here it was not necessary to include exogenous lymphokines to induce adequate CTL activity. The addition of large amounts of rhIL2 (Figs. 3.13 and 3.14), shown previously to induce proliferation of activated equine T cells, had the effect of increasing the killing of autologous infected target cells by CTL at low effector:target ratios. However, the specificity and genetic restriction of the resultant

CTL remained unchanged.

High levels of cold target inhibition of effector cell killing was achieved using autologous, infected PWM blast cells. Autologous mock-infected cold targets inhibited killing of CTL by a maximum of 20% indicating that either a second population of non-specific cytolytic cells had developed or that the cold targets were preventing efficient contact between the CTL and the labelled targets. The development of non-specific killer cells in the induction cultures was unlikely as autologous mock-infected, allogeneic infected and allogeneic mock-infected target cells were not efficiently killed in any CTL assays performed to date (see Figs. 3.1, 3.2, 3.6 and 3.7). The partial inhibition of killing using allogeneic, infected cold targets (see Fig. 3.5b, open squares) again indicates that, non-specific blocking of efficient contact between CTL and labelled target cells occurred. The induction of non-specific killers in induction cultures of the LAK cell type did not occur as CTL did not kill the LAK cell sensitive EqT8888 cell line (data not shown). Because of recent results presented at the second Equine leukocyte antigen workshop (Lake Tahoe, USA, 1995), it should be possible to measure the proportion of NK cells induced by depletion of the effector cell population using monoclonal antibodies specific for human CD16. This molecule, present on the surface of NK cells, is an Fc receptor and recognises approximately 10% of equine PBMC (J. Kydd, Animal Health Trust, Newmarket, personal communication). Another explanation for the inhibition of CTL activity by allogeneic infected cold targets (Fig. 3.5b, open squares) is that the effector cells may be MHC class II restricted. Although the two horses used in

this experiment were shown to be ELA class I disparate (as demonstrated by IEF of immune precipitated ELA antigens), they may have shared class II alleles. Whatever the mechanism behind the observed allo-specific inhibition, it is clear that genetically restricted CTL comprise the majority of the effector cell population because autologous cells were much more efficient as cold targets compared with allogeneic cells.

Direct, evidence of the prevalence of $CD8^+$ CTL in the induced population was obtained from the T cell depletion experiment (Fig. 3.15). The high levels of specific, genetically restricted cytotoxicity observed in the CD4-depleted but not in the CD8-depleted populations indicates a major role for $CD8^+$ T cells in the killing of EHV-1 infected target cells *in vitro*. These results agree with those of Allen *et. al.* (1995). Depletion rather than positive selection with monoclonal antibodies was used in this experiment in order to avoid modulation of CTL activity due to the binding of antibodies to cell surface molecules.

The mechanisms involved in the development of EHV-1 specific effector CTL from CTLp using live virus are not obvious from the studies carried out. However, the observation that, in resting PBMC populations, up to about 20% of the cells are infectable with EHV-1 strain Ab4 (see chapter 2) means that these cells may act as antigen presenting cells for CTLp in induction cultures. The presence of neutralising antibody in the autologous serum prevents the release of live virus which may inhibit the proliferation of CTLp. The presence of activated, T cells of both the CD4 (17% infected) and CD8

(24% infected) phenotype (Allen *et. al.*, 1995) precluded the need for the addition of exogenous lymphokines.

Using this system it has been relatively easy to demonstrate high levels of specific, genetically restricted cytotoxicity in adult, immune animals. However, these experiments, whilst valuable in the development of CTL assay techniques, do not tell us much about the role of EHV-1 specific CTL in field infections. In order to begin to address this point, a wider range of horses, including foals of various ages, were studied. As outlined in chapter 1, it is known that foals become infected with EHV-1 or EHV-4 early in life, thus developing a degree of immunity to each virus (reviewed in Allen & Bryans, 1986). This immunity has been characterised by the development of specific virus neutralising antibodies. However, until now, it has not been possible to assess whether virus-specific CTL develop after these early infections. The data presented in Figures 3.8 and 3.9 strongly suggests that the initial infection of young animals occurs before 10 months of age and that this primary infection results in the development of a degree of CTL activity. It is clear from Figures 3.8a, 3.9a and 3.9b that the animals represented here, one 18 day old foal, one 3 week old foal and one 4 month old foal, had no detectable CTL activity whilst the animal represented in Figure 3.8b, a 10 month old foal, had developed significant, measurable CTL. The measurement of weak CTL activity after a true primary infection (Fig. 3.10), indicates that this arm of the immune response to EHV-1 is not vigorous. The necessity of laborious and expensive production of small numbers of SPF animals (Chong *et. al.*, 1991) in order to study primary

responses to equine viruses was questioned by data presented in Figures 3.8 and 3.9. These data suggest that young, conventionally reared animals did not become infected with either EHV-1 or EHV-4 until at least 4 months of age. This raises the possibility that with careful management and frequent immunological monitoring, these ponies could be used for the study of primary infections instead of SPF foals.

In older animals, repeated infection with EHV-1 and/or EHV-4 results in the development of cross-reactive CTL (see Fig. 3.11 and Allen *et. al.*, 1995). Results presented in Fig. 3.11 show that CTL induced from different immune animals reacted differently to various isolates of EHV-1 and EHV-4. Figure 3.12 shows that this differential pattern of response persisted over a long period and between different assays. This observation may reflect differences in the infection histories of the ponies or differences in the capacity of each pony to respond to particular epitopes on each of the viruses. Another possibility is that a reduced reaction to a particular virus (*e.g.* EHV-1 Ab4 in Fig. 3.12) may have occurred due to a hyperactivation of cells in the *in vitro* culture system which subsequently depleted nutrients from the culture medium and hence produced a lower than expected % specific cytotoxicity value. More work is needed to characterise the nature of the cross-reaction and differential reactivity of CTL. The re-stimulation of cross-reactive CTL with EHV-1 and EHV-4 is analogous to the situation in which HSV induces both types of CTL activity in mice after infection (Eberle *et. al.*, 1981, Carter *et. al.*, 1982). Cross-reactive and type-specific cytolytic T cell clones against HSV-1 and HSV-2 have also been demonstrated in humans (Yasukawa &

Zarling, 1984).

The preliminary studies presented in section 3.3.8 suggest that a proportion of EHV-1 induced CTL recognise immediate early proteins. Further analyses of this type will determine which antigens from EHV-1 and EHV-4 are recognised by virus-specific CTL.

In order to determine the cross-reactive or type-specific nature of the anti EHV CTL response the derivation of T cell clones or the use of limiting dilution analysis (LDA) is necessary. The cloning of equine EHV specific CTL has proven difficult (D. Hannant, Animal Health Trust, Newmarket, personal communication), however, an LDA assays for the measurement of anti EHV-1 and EHV-4 CTLp frequencies has been developed and is reported in chapter 5.

4. T cell proliferative responses

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4.1. Introduction

The roles of antibody and *in vitro* proliferation of equine PBMC to inactivated EHV-1 (iEHV-1) as indicators of immunity to EHV-1 have been widely studied (Stokes *et. al.*, 1991, Thomson & Mumford, 1977, Dutta *et. al.*, 1980). These studies (see also Mumford *et. al.*, 1987, Hannant *et. al.*, 1993) have shown that high levels of virus neutralising antibodies do not prevent infection of horses with EHV-1. The conclusions from T cell proliferation studies have been that there was great variation in responses between individuals after infection with EHV-1 and that the levels of lymphocyte stimulation do not correlate with immunity. Evidence for effective immunity to EHV-1 has come from experiments in which animals were repeatedly infected with the virus. For example, Allen and Bryans (1986) showed that intranasal infection of seronegative weanling foals with EHV-1 and subsequent re-infection four weeks later with either EHV-1 or EHV-4 resulted in immunity against challenge with the homotypic virus but not with the heterotypic virus. Further, it was shown that after three sequential infections with either EHV-1 or EHV-4 (second infection four weeks after primary infection and third infection five months later), foals were resistant to challenge with the heterotypic virus. In contrast to these results, Edington (1990) showed that two consecutive EHV-4 infections did not prevent infection or significantly reduce the duration of nasal shedding after EHV-1 challenge. However, two consecutive infections of yearling ponies with EHV-1 spaced at five month intervals protected against a tertiary infection five months later with EHV-4. Despite the differences seen in these studies, which may have been due to differences in

experimental design, both authors concluded that multiple infections with EHV-4 may give some protection against a potentially more serious EHV-1 infection. The protective mechanisms were not discussed in depth by either author. Conclusions would have been difficult to make given that the only immunological parameter measured was the level of circulating antibody. From these studies, it is clear that infection by either virus does not induce complete immunity against both EHV-1 and EHV-4. Moreover, the effector mechanisms responsible for resistance are as yet, not understood. Because serum antibody appears to play no role in protection, it is likely that cellular immune responses have important functions in immunity to EHV.

Although there have been several studies documenting *in vitro* proliferative responses of PBMC to viral antigen after EHV infection, there is no evidence to suggest that high levels of PBMC proliferation correlate with immunity to infection. Therefore, a study was carried out to determine if *in vitro* measures of T cell function would be of value in this respect. T cell function was assessed using polyclonal activation of PBMC with mitogens and viral antigens, and by measuring the precursor frequency of EHV-1-specific proliferative T cells using limiting dilution analysis (LDA). The objectives were to measure T cell responses in horses before and after EHV-1 infection and by observation of the virological outcome of infection, to determine if these responses correlated with immunity. Four horses were examined in this study, 2 had received multiple previous experimental infections with EHV-1, whilst the other 2 had not.

4.2. Materials and Methods

4.2.1. Animals and infection

Four pregnant Welsh mountain pony mares (#24, #26, #219 and #302) were infected with $10^{8.4}$ $TCID_{50}$ of a plaque-purified strain of EHV-1 Ab4/13 (Telford *et. al.*, 1992) by exposure to a nebulised aerosol as described (Mumford *et. al.*, 1990 and 1994). This isolate has been shown to share the pathogenic characteristics of an earlier passage strain (Ab4/11 grown in RK13 cells) in specific pathogen free (SPF) foals (Gibson *et. al.*, 1992b). The mares were infected in the ninth month of gestation. Mares #24 and #26 were obtained from a supplier and for 9 months before infection did not show any serological evidence of infection or recrudescence of EHV-1 or EHV-4 (Thomson *et. al.*, 1976b). Animals with a similar history have been shown to be susceptible to EHV-1 infection (Mumford *et. al.*, 1994). Mare #219 had received one previous experimental infection with EHV-1/Ab4 whilst pregnant and one whilst non-pregnant, and #302 had received one previous experimental infection with EHV-1/Ab4 whilst pregnant within the previous two years.

4.2.2. Sampling, serology and virus isolation

After infection, the mares were sampled regularly for the determination of serum complement-fixing (CF) antibody (Thomson *et. al.*, 1976a) and virus isolation from nasal swabs and heparinised blood (Mumford *et. al.*, 1987). Rectal temperatures were recorded daily for two weeks.

4.2.3. Lymphoproliferation assays

Equine PBMC were isolated from whole blood using Ficoll Hypaque (Pharmacia Biotech Ltd, Milton Keynes, Beds.) as described in section 3.2.1. They were resuspended in complete RPMI (cRPMI), at a concentration of $2x10^6 ml^{-1}$.

Aliquots of PBMC (100µl) were distributed into round bottomed microwell plates (Falcon, Becton Dickinson Labware, Cowley, Oxford) containing titrated amounts of the mitogens Con A (2.5μgml^{-1} to 10μgml^{-1}) or PHA (0.625μgml^{-1} to 2.5μgml^{-1}) in 100µl volumes, giving a total culture volume of 200µl. Antigen-specific proliferation was assayed using iEHV-1 antigen (virus heat-inactivated at 60°C for 30 min) because preliminary experiments showed that PBMC did not respond to live virus antigen *in vitro* (data not shown). Moreover, live EHV-1 virus was shown to inhibit proliferation stimulated by mitogen or third party antigens (see below). Unstimulated control PBMC were set up in cRPMI only.

The mitogen assays were incubated for 48 hours and the antigen cultures were incubated for 120 hours at 37°C in an atmosphere of 5% CO_2 in air. Subsequently all cultures were pulsed with 0.5µCi of tritiated thymidine per well for 18 hours before harvesting on to glass fibre filters using a Skatron cell harvesting system (Skatron Ltd, Newmarket, Suffolk.). Scintillation fluid (Ultima Gold. Canberra Packard, Pangbourne, Berks.) was added to each filter disk (1ml per disk) and the radioactivity was counted in a scintillation

counter (Packard). Results were presented as counts per minute (cpm) and stimulation indices (SI = cpm stimulated well/cpm control well).

To demonstrate the effects of culturing live or iEHV-1 on the proliferation of viable PBMC stimulated with mitogens or third party antigens (Keyhole Limpet Haemocyanin-KLH), variations in the basic lymphoproliferation assay were made. In these experiments, live or iEHV-1 (strain Ab4/13) was titrated in cRPMI in microtire plates and $2x10^5$ PBMC containing dilutions of mitogen (PHA) or KLH were added. In the experiments with KLH, the PBMC were obtained from a horse which had been immunised twice with 100μg KLH in an aluminium hydroxide adjuvant (alhydrogel). The virus used was titrated in log steps from $1x10^{7.6}$ to $1x10^{1.6}$ $TCID_{50}ml^{-1}$, the PHA was used at 2.5, 1.25, 0.625 and $0\mu gml^{-1}$ and the KLH was used at 25, 12.5, 6.25 and $0\mu gml^{-1}$ in a total culture volume of 200μl. Again, all cultures were pulsed with 0.5μCi of tritiated thymidine* per well for the final 18 hours of the incubation period before harvesting. Comparisons were made between the pre- and post-infection cpm and SI values using Student's *t* test.

4.2.4. Proliferative limiting dilution analysis

A proliferative LDA assay was developed based on the assay described by Fischer *et. al.* (1991). An explanation of the theory behind LDA is provided in Appendix B.

* 70 - 86 Ci/mmol.

Varying numbers of responder PBMC were cultured in 200µl volumes in the presence of 20,000 autologous cells which had been treated with mitomycin C* (Sigma) and a final dilution of 1/500 of iEHV-1 derived from pelleted virus (originally $10^{10.0}$ $TCID_{50}$ ml^{-1}). The cultures were set up in round bottomed microtitre plates. As negative controls, cultures were set up containing mitomycin C treated PBMC with antigen but no responder cells. Each responder cell concentration was set up using a replicate number of 24 as recommended (Fazekas de St.Groth, 1982).

The LDA cultures were incubated at 37°C for 6 days including an 18 hour pulse with 0.5µCi per well of tritiated thymidine and cultures were harvested and counted as described for the conventional lymphoproliferative assays.

Positive wells were identified as those in which the measured cpm were greater than 3 standard deviations above the mean cpm in the control wells. The data for each assay were tabulated and the frequency of responding cells was estimated using the computer program designed by Strijbosch *et. al.* (1988). The Jacknife version of the maximum likelihood method was used for statistical analysis as recommended (Strijbosch *et. al.*, 1987). Any frequency determinations which generated chi squared goodness-of-fit statistics greater than published values, using the dilution number minus one for the degrees of freedom, were rejected as not satisfying single hit kinetics (Bouma et. al. 1992). The frequencies of responding cells in any two populations were considered significantly different if their 95% confidence limits did not overlap (Fischer et. al. 1991).

* 50µg.

The statistical analysis is explained in more depth in appendix B. Examples of the data obtained from these experiments are shown in appendix C.

4.3. Results

4.3.1. The effect of EHV-1 on *in vitro* immune functions

During EHV-1 viraemia the *in vitro* proliferation of PBMC in response to mitogens is suppressed (Hannant *et. al.*, 1991. see also figures 4.4, 4.5, 4.6 and 4.7). This may be due to the suppressive effects of cell-associated EHV-1 antigen present during viraemia. Experiments were carried out in order to determine the effect of live or iEHV-1 on *in vitro* proliferation of equine PBMC. Live or iEHV-1 were included, at various concentrations, in assays of proliferation to mitogen and specific antigen.

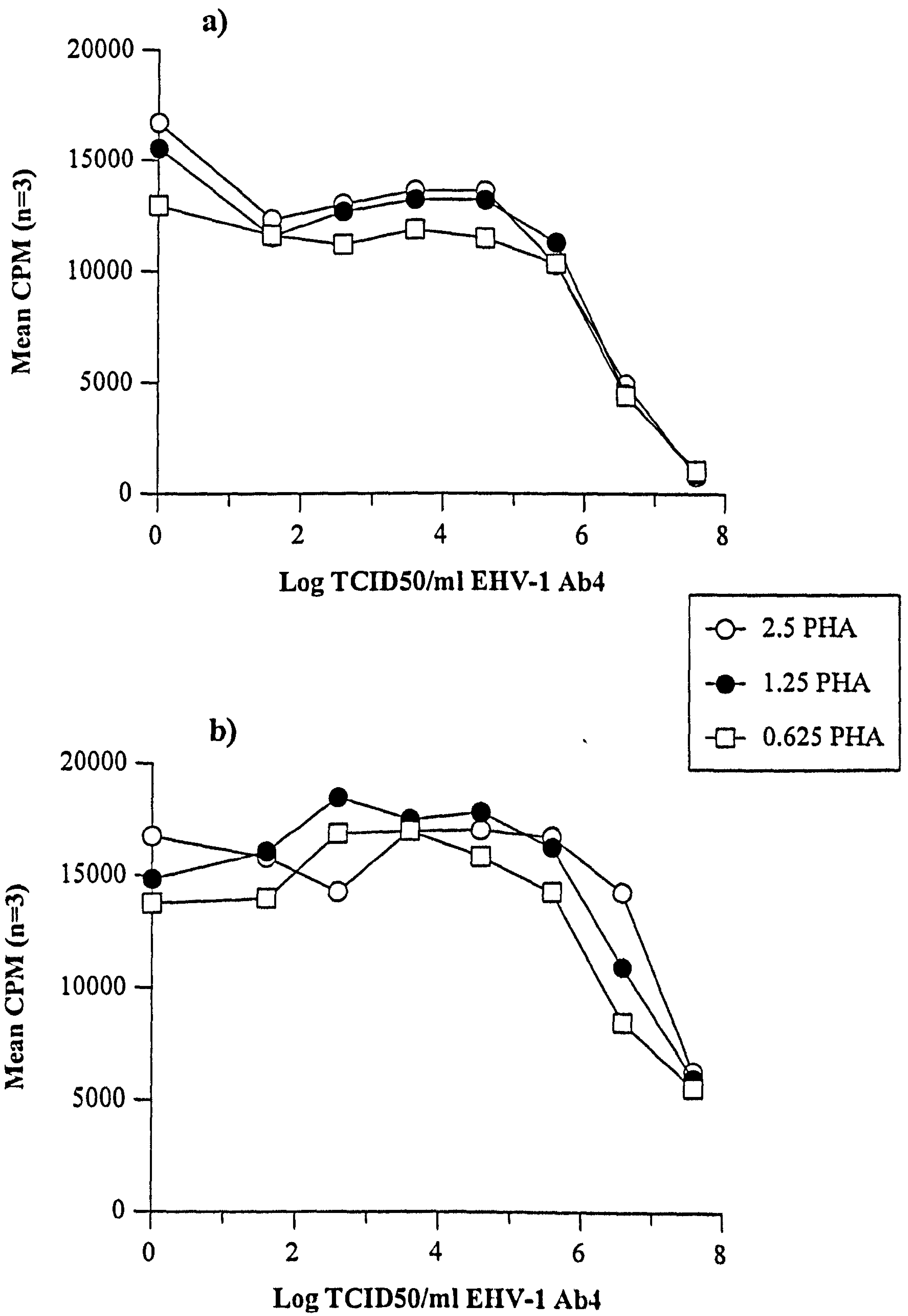
Figure 4.1a shows that live virus suppressed the response of equine PBMC to PHA. At virus concentrations between $1x10^{1.6}$ and $1x10^{5.6}$ $TCID_{50}ml^{-1}$, equivalent to multiplicities of infection (moi) of between 0.00002 and 0.2, the mean cpm of the PHA response was significantly ($p<0.05$) reduced by approximately 20%. When the $TCID_{50}$ of the virus exceeded $1x10^{5.6}$ $TCID_{50}ml^{-1}$ the mean cpm decreased further until at an moi of 20 the proliferative response was reduced by approximately 95% ($p<0.001$).

Figure 4.1. The effect of live and inactivated EHV-1 on *in vitro* mitogen induced proliferation of equine PBMC.

a) Live EHV-1
b) Heat inactivated EHV-1

Significant reductions in mean cpm occurred at:

a) Between $1x10^{1.6}$ and $1x10^{5.6}$ $TCID_{50}$ $p<0.05$.
   Between $1x10^{6.6}$ and $1x10^{7.6}$ $TCID_{50}$ $p<0.001$.
b) Between $1x10^{1.6}$ and $1x10^{5.6}$ $TCID_{50}$ no significant decrease.
   Between $1x10^{6.6}$ and $1x10^{7.6}$ $TCID_{50}$ $p<0.01$.



a)
Mean CPM (n=3)
20000
15000
10000
5000
0
0
2
4
6
8
Log TCID50/ml EHV-1 Ab4
2.5 PHA
1.25 PHA
0.625 PHA
b)
Mean CPM (n=3)
20000
15000
10000
5000
0
0
2
4
6
8
Log TCID50/ml EHV-1 Ab4

When iEHV-1 was used in an identical experiment (Fig. 4.1b) the mean cpm remained at or above the level of proliferation induced in the absence of virus between virus concentrations equivalent to $1x10^{1.6}$ and $1x10^{5.6}$ $TCID_{50}ml^{-1}$. Mean cpm values above this level in this experiment reflected the contribution of antigen-specific proliferation to the total response. When the virus concentration was increased further, some suppression of the mitogenic response was seen (up to approximately 37% at an moi of 20), however, the reduction in mean cpm caused by iEHV-1 was much lower than that caused by live EHV-1.

When the effects of live EHV-1 on antigen-specific responses to the third party antigen KLH were measured, the responses were, again suppressed. Figure 4.2a shows the reduction of the mean cpm in response to KLH caused by live EHV-1. Between virus concentrations of $1x10^{1.6}$ and $1x10^{5.6}$ $TCID_{50}ml^{-1}$ the reduction of mean cpm was relatively constant (significant reductions occurring at a KLH concentration of $12.5\mu gml^{-1}$ only). At higher concentrations of live virus, the mean cpm values decreased to significantly ($p<0.01$). When iEHV-1 was used, there was no significant reduction of the mean cpm (fig. 4.2b).

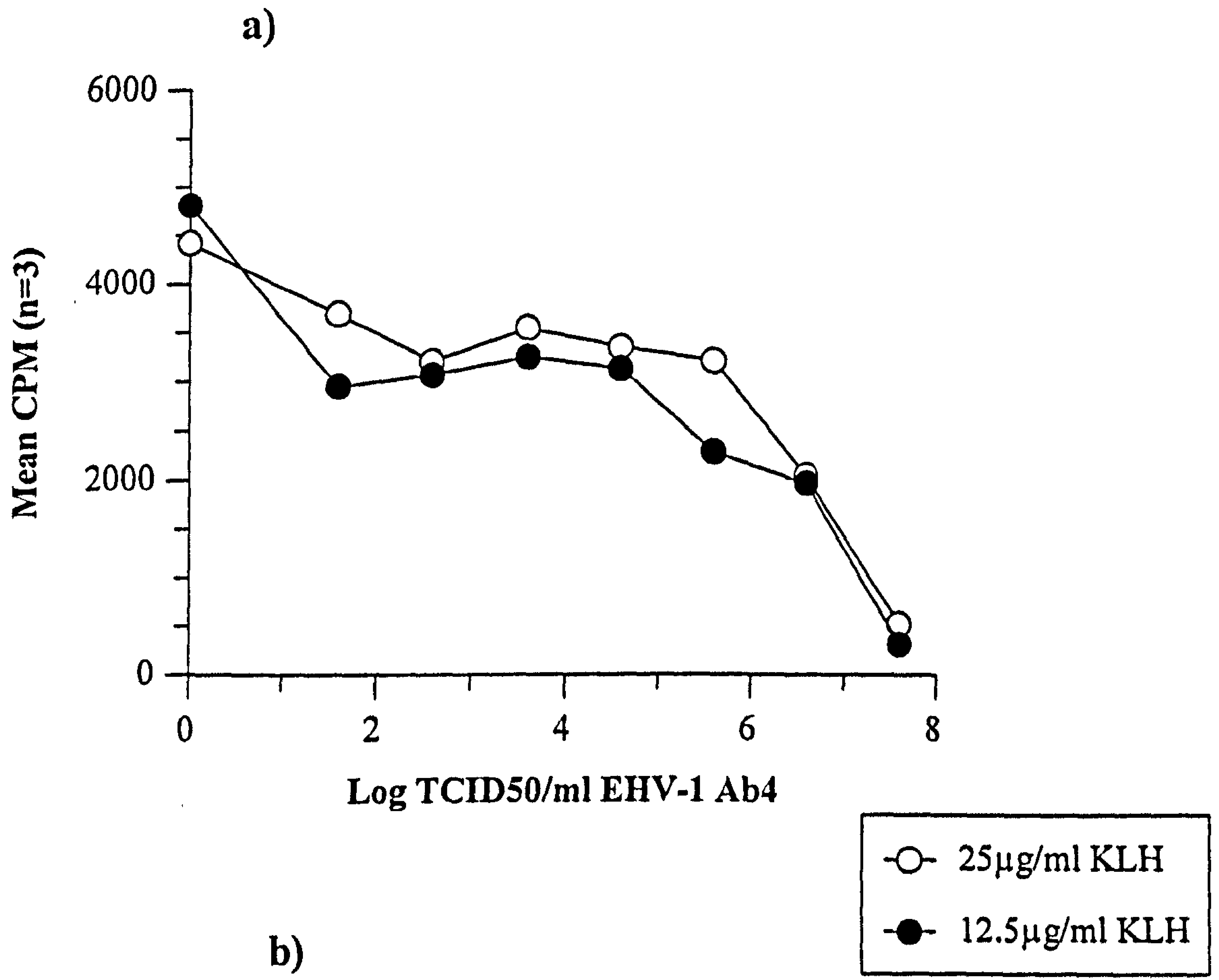
Further analysis of the data from the KLH experiment revealed that when no KLH was included in the proliferation assay significant EHV-1-specific proliferative response occurred to iEHV-1 at concentrations above $1x10^{6}$ $TCID_{50}ml^{-1}$. The results from this assay (Figure 4.3) showed that live EHV-1 did not stimulate a proliferative response

Figure 4.2. The effect of live and inactivated EHV-1 on *in vitro* antigen induced proliferation of equine PBMC.

a) Live EHV-1
b) Heat inactivated EHV-1

Significant reductions in mean cpm occurred at:

a) Between $1x10^{1.6}$ and $1x10^{5.6}$ $TCID_{50}$ $p<0.05$.
   Between $1x10^{6.6}$ and $1x10^{7.6}$ $TCID_{50}$ $p<0.02$.
b) Between $1x10^{1.6}$ and $1x10^{7.6}$ $TCID_{50}$ no significant decrease.


a)
6000
4000
2000
0
Mean CPM (n=3)
0
2
4
6
8
Log TCID50/ml EHV-1 Ab4
25μg/ml KLH
12.5μg/ml KLH

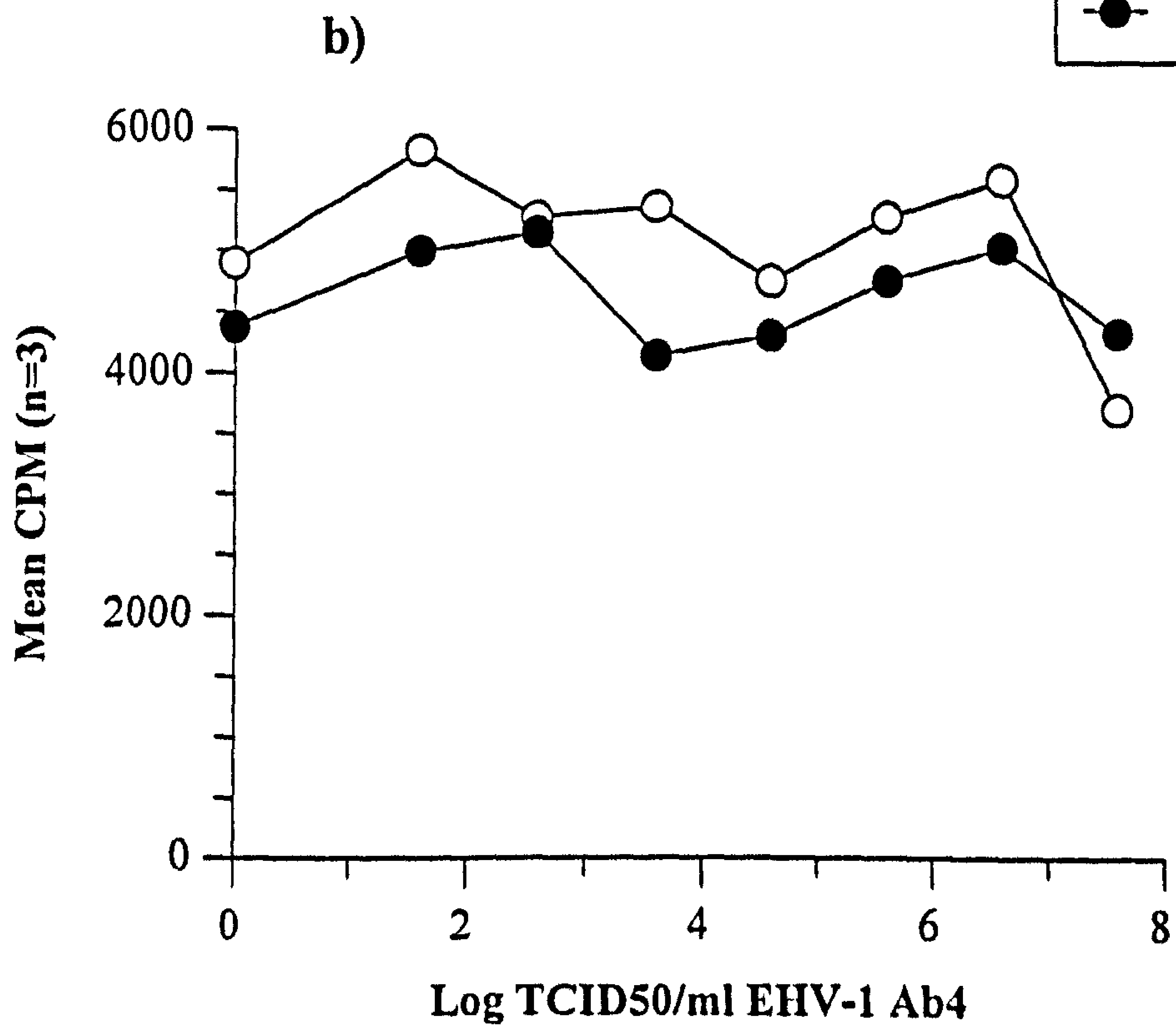
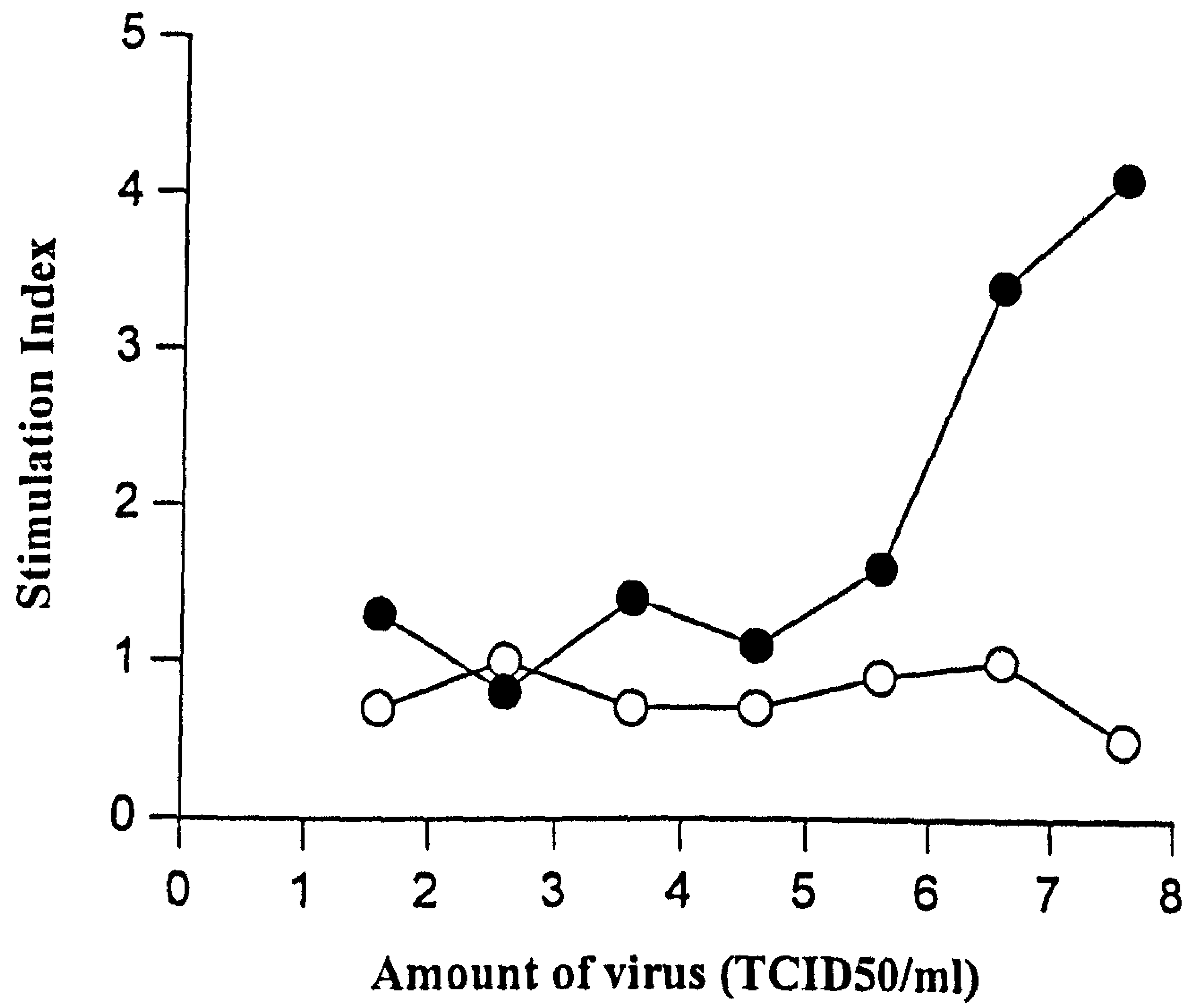

b)
6000
4000
2000
0
Mean CPM (n=3)
0
2
4
6
8
Log TCID50/ml EHV-1 Ab4

Figure 4.3. Proliferative response of equine PBMC induced with live or heat inactivated EHV-1.

open circles = live EHV-1
closed circles = heat inactivated EHV-1



5
4
3
2
1
0
Stimulation Index
0
1
2
3
4
5
6
7
8
Amount of virus (TCID50/ml)

above background levels. These results showed that it was not possible to use live EHV-1 to study virus-specific T cell proliferation. Hence, inactivated virus was used for all subsequent antigen-specific proliferation assays. Additionally, the data suggest that productive virus infection of PBMC during periods of cell-associated viraemia has the potential to suppress *in vivo* antigen specific proliferative responses.

4.3.2. Responses to challenge infection with EHV-1/Ab4/13

Of the four mares infected with EHV-1/Ab4/13, two (#24 and #26) had unknown previous infection histories, although they had no serological evidence of infection for 9 months prior to the infection study. Of the other two mares, #219 had received one previous experimental infection with EHV-1/Ab4 whilst pregnant and one whilst non-pregnant, and #302 had received one previous experimental infection with EHV-1/Ab4 whilst pregnant within the previous two years (see materials and methods for the timings of the previous infections).

A summary of the clinical and virological responses of mares #219 and #302 to their first previous experimental infection (referred to as the initial infection) can be seen in Table 4.1. The clinical and virological responses of all four mares to EHV-1/Ab4/13 infection are shown in Table 4.2a.

Infection of mares #24 and #26 with EHV-1/Ab4/13 resulted in pyrexia (a body temperature of above 102°F or 38.9°C) in both animals for 2 days. However, no

Table 4.1. Clinical and virological responses following first experimental infection with EHV-1 Ab4/8.

a) Duration of clinical and virological signs.

Mare	Pyrexia	Duration (Days) Viraemia	Nasal VI
219	2	4	3
302	1	8	6

Nasal VI = Virus isolation from nasal swab extracts.
Viraemia = Virus isolation from heparinised blood.
Both ponies seroconverted as measured by CF.

b) Level of virus in nasal swabs.

	Days after aerosol infection									
	1	2	3	4	5	6	7	8	9	10
219	ND	2.6	2.3	2.9	-	-	-	-	-	-
302	3.6	4.1	3.6	4.1	3.6	2.4	-	-	-	-

Results presented as $TCID_{50}$ ml^{-1} nasal swab extract
ND = not determined

Table 4.2. Clinical and virological responses following infection with EHV-1 Ab4/8.

a) Duration of clinical and virological signs.

Mare	Duration (Days) Pyrexia	Viraemia	Nasal VI
24	2	5	4
26	2	4	7
219	0	2	3
302	0	3	1

Nasal VI = Virus isolation from nasal swab extracts.
Viraemia = Virus isolation from heparinised blood.
All ponies seroconverted as measured by CF.

b) Level of virus in nasal swabs.

	Days after aerosol infection									
	1	2	3	4	5	6	7	8	9	10
24	ND	5.6	4.6	3.3	3.6	-	-	-	-	-
26	3.1	4.6	5.3	4.8	3.8	4.1	2.1	-	-	-
219	1.3	4.6	-	1.3	-	-	-	-	-	-
302	3.6	-	-	-	-	-	-	-	-	-

Results presented as $TCID_{50}$ ml^{-1} nasal swab extract. ND = not determined

pyrexia was seen in mares #219 and #302 in contrast to their initial infections (Table 4.1a). The pyrexic responses of all four mares were markedly different to the well-documented biphasic pyrexic response seen after infection of "susceptible" mares (Mumford *et. al.*, 1994). This suggests that mares #24 and #26 had a degree of immunity to EHV-1 due to previous exposure. However, this degree of immunity was not as effective as that induced by previous experimental infections in #219 and #302, as these animals were protected against pyrexia after challenge.

Mares #219 and #302 excreted less virus for a shorter time from the nasopharynx when compared with mares #24 and #26 (see Table 4.2b). After EHV-1 Ab4/13 challenge both mares #24 and #26 showed signs of virus-induced disease. For example, mare #24 became ataxic between 9 and 11 days after infection whilst mare #26 gave birth to a weak foal, which died soon after birth, 15 days after infection. Mares #219 or #302 showed no such disease and produced healthy foals at full-term. After their initial infection with EHV-1 Ab4, mare #219 aborted whilst mare #302 carried her foal to term despite the fact that she excreted 100 fold more virus from the nasopharynx than #219 and showed a longer period of cell associated viraemia (see Table 4.1). All mares seroconverted as measured by the CF assay (data not shown).

4.3.3. Bulk culture lymphoproliferative responses

Bulk culture, mitogen-induced proliferation of PBMC from mares #219 and #302 showed that the rapid reduction in responsiveness to PHA and Con A after EHV-1 infection,

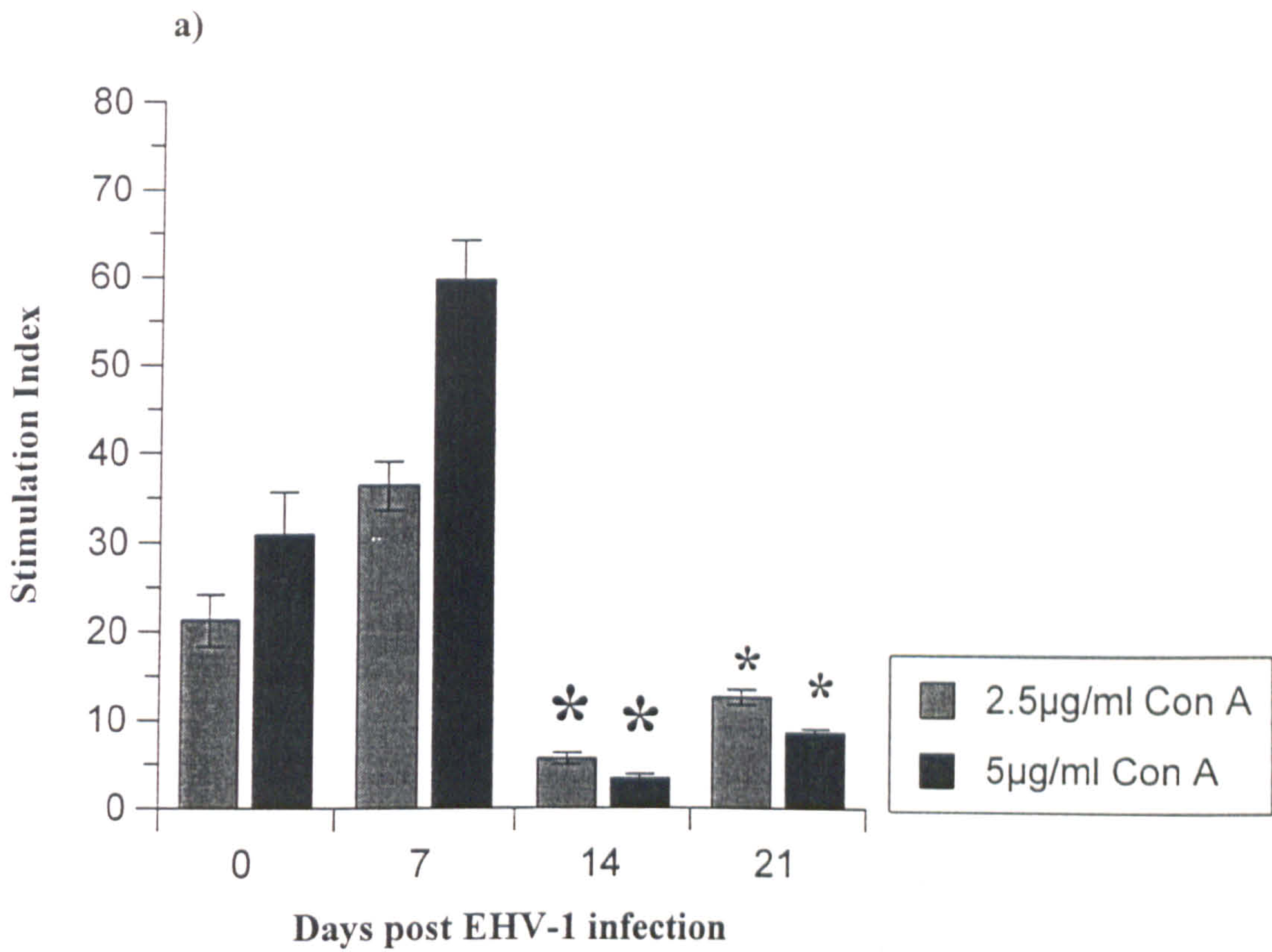
reported by Hannant *et. al.* (1991), was delayed in these animals (see Figures 4.4 and 4.5) when stimulation index was used. SIs were equivalent to or slightly above pre-infection levels 7 days after infection but were reduced significantly 14 ($p<0.001$) and 21 ($p<0.01$) days after infection. However, when mean cpm were analysed, no reduction in the response of PBMC from either mare was seen (Table 4.3). The reduction in the SI values on days 14 and 21 post-infection were entirely due to increases in the background cpm on those days.

Mitogen-induced proliferation of PBMC from mares #24 and #26 was monitored up to day 28 after infection. Pre-infection SIs were similar to those of mares #219 and #302. One day after infection responses of PBMC to both PHA and Con A were significantly lower ($p<0.001$ and 0.01 respectively, see Figures 4.6 and 4.7) than pre-infection SIs, except when PBMC from mare #26 were stimulated with Con A (see Figure 4.7a). The response was further reduced in both animals 7 days after infection ($p<0.001$). These reductions in SI values after infection with EHV-1 were not due to increases in the background cpm (Table 4.4). Mitogen-induced SI values from these ponies were significantly increased above pre-infection levels 14 and 28 days after infection. However, when mean cpm were analysed, there was only a significant increase above pre-infection cpm values on day 14 post-infection. The significance of the observed reductions in the responsiveness of PBMC to mitogens and the subsequent enhancement of the response in these animals after EHV-1 infection is unclear, although a link with viraemia seems likely.

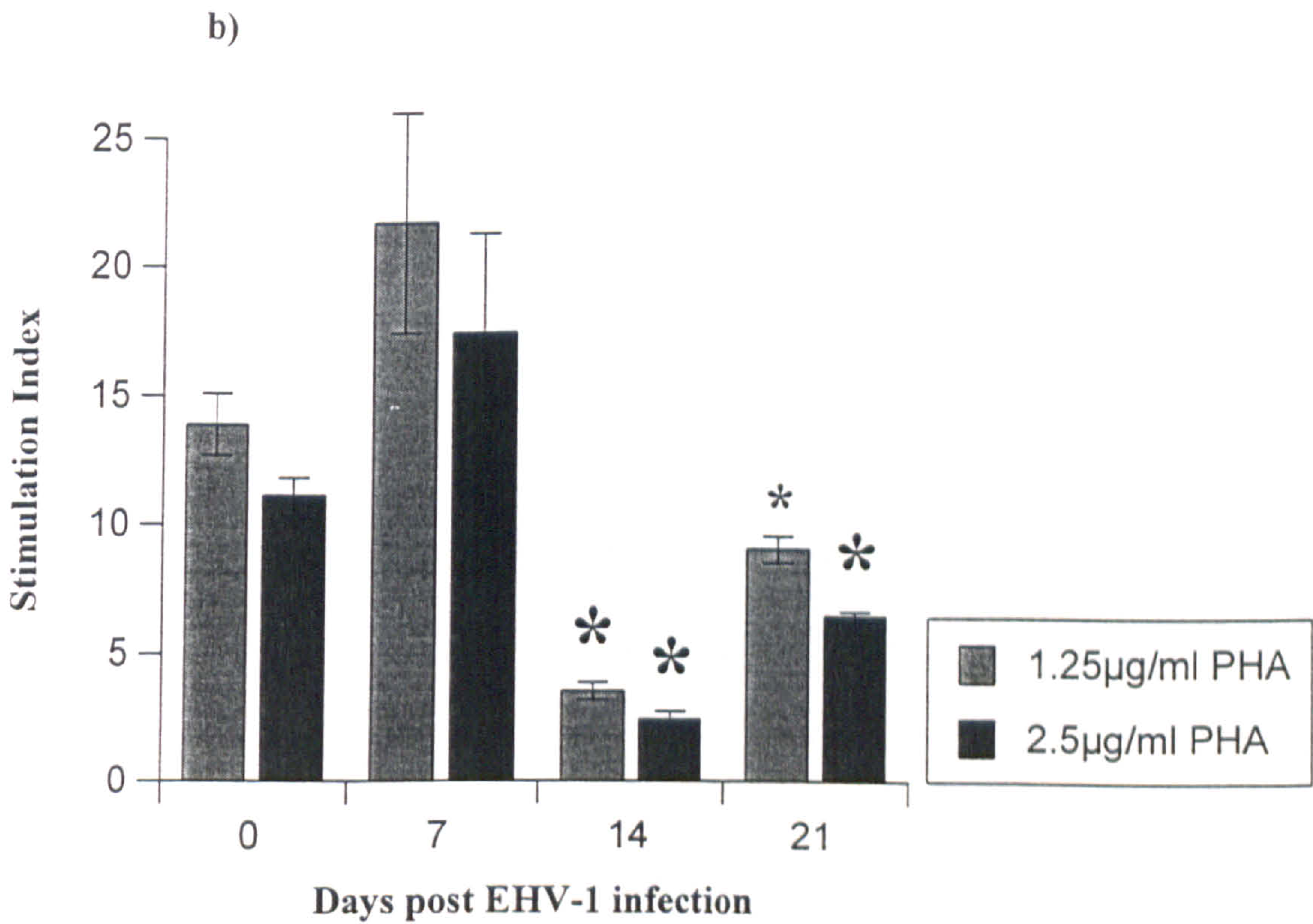
Figure 4.4. Mitogen-induced proliferation of PBMC from mare #219 after EHV-1 infection.

a) Con A induced proliferation
b) PHA induced proliferation

a)



b)



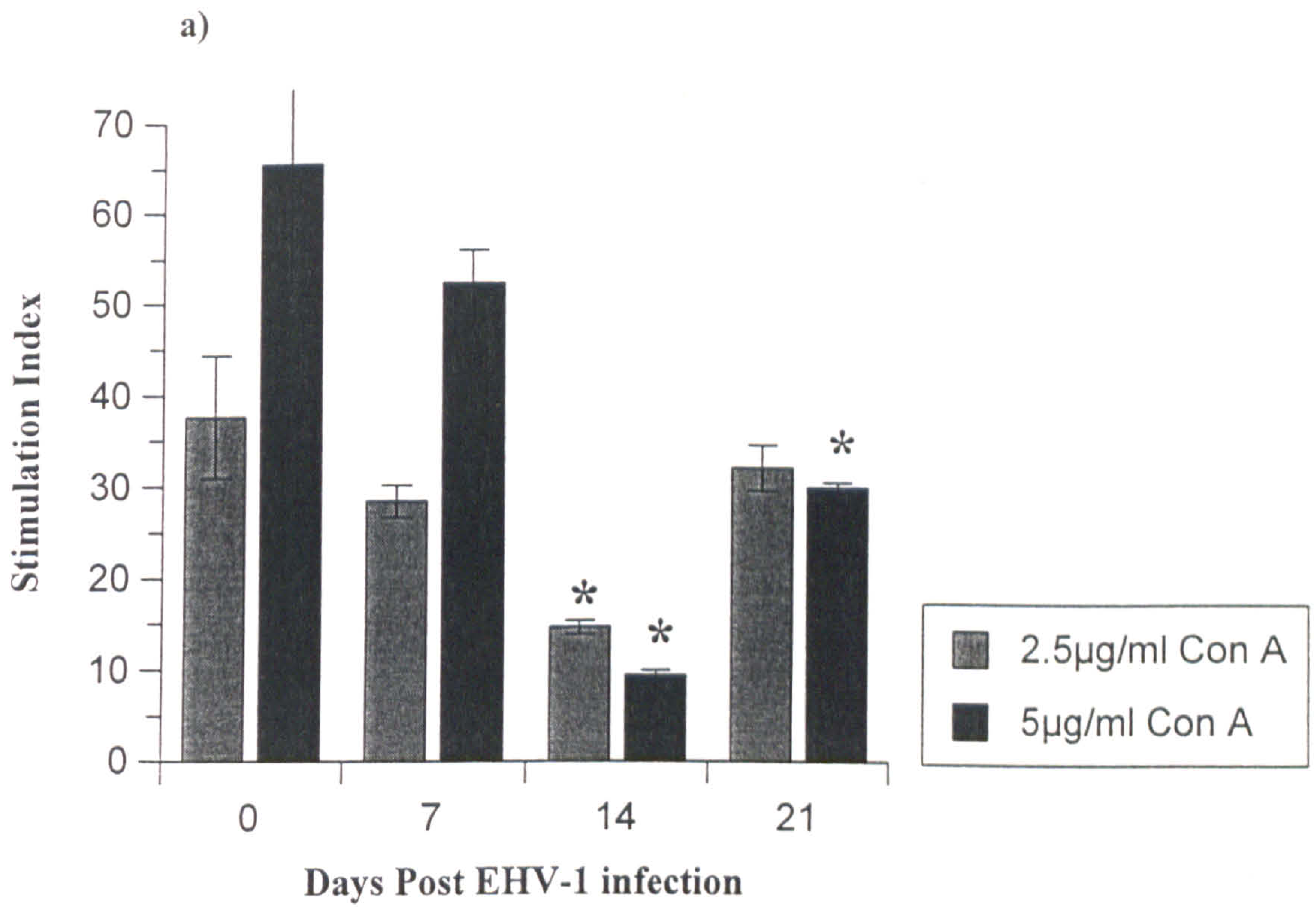
* = a significant drop in SI p<0.001.

* = a significant drop in SI p<0.01.

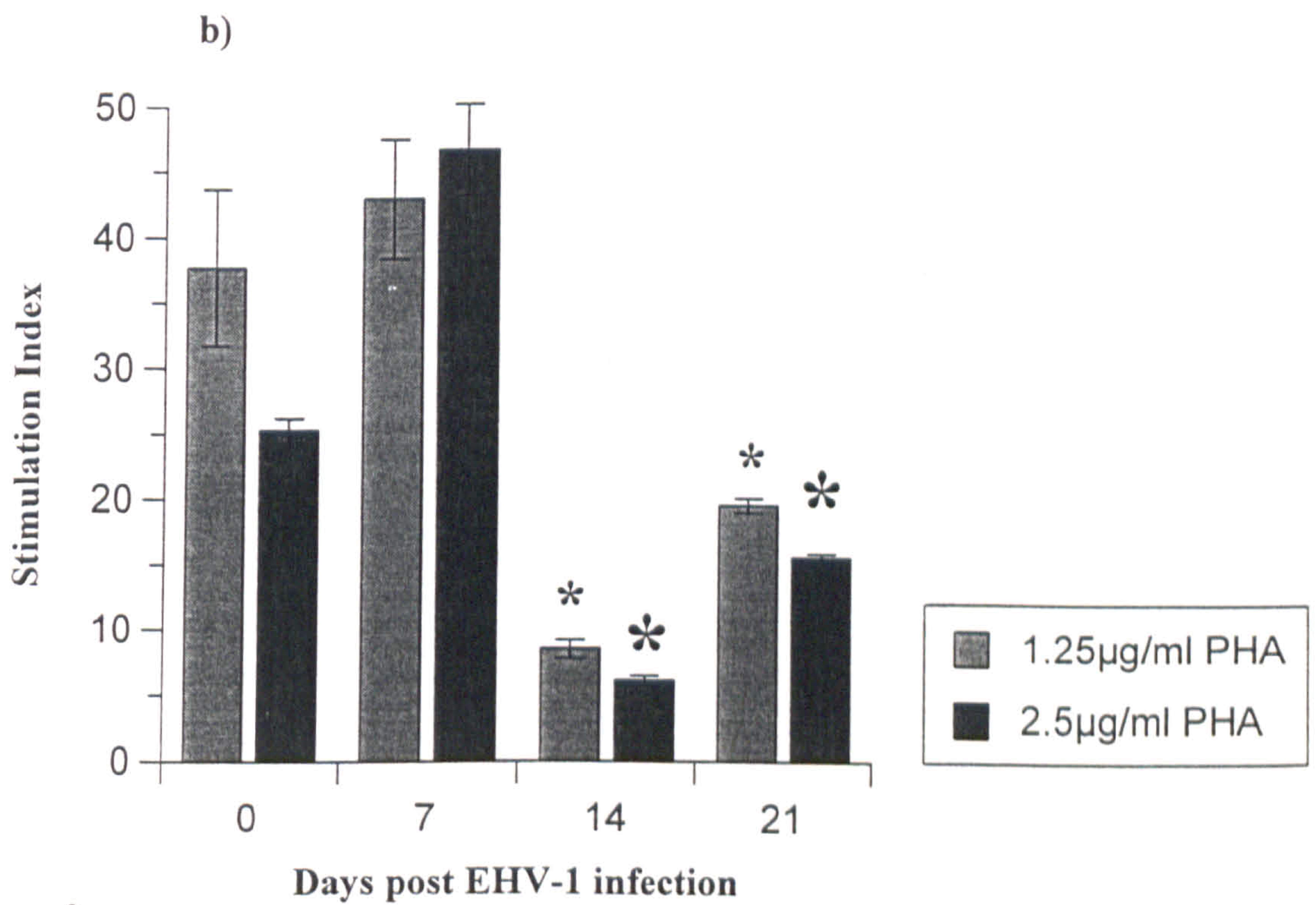
Figure 4.5. Mitogen-induced proliferation of PBMC from mare #302 after EHV-1 infection.

a) Con A induced proliferation
b) PHA induced proliferation

a)



b)



* = a significant drop in SI $p<0.001$.

* = a significant drop in SI $p<0.01$.

Table 4.3. Proliferation of PBMC from mares #219 and #302 in response to mitogens before and after infection with EHV-1.

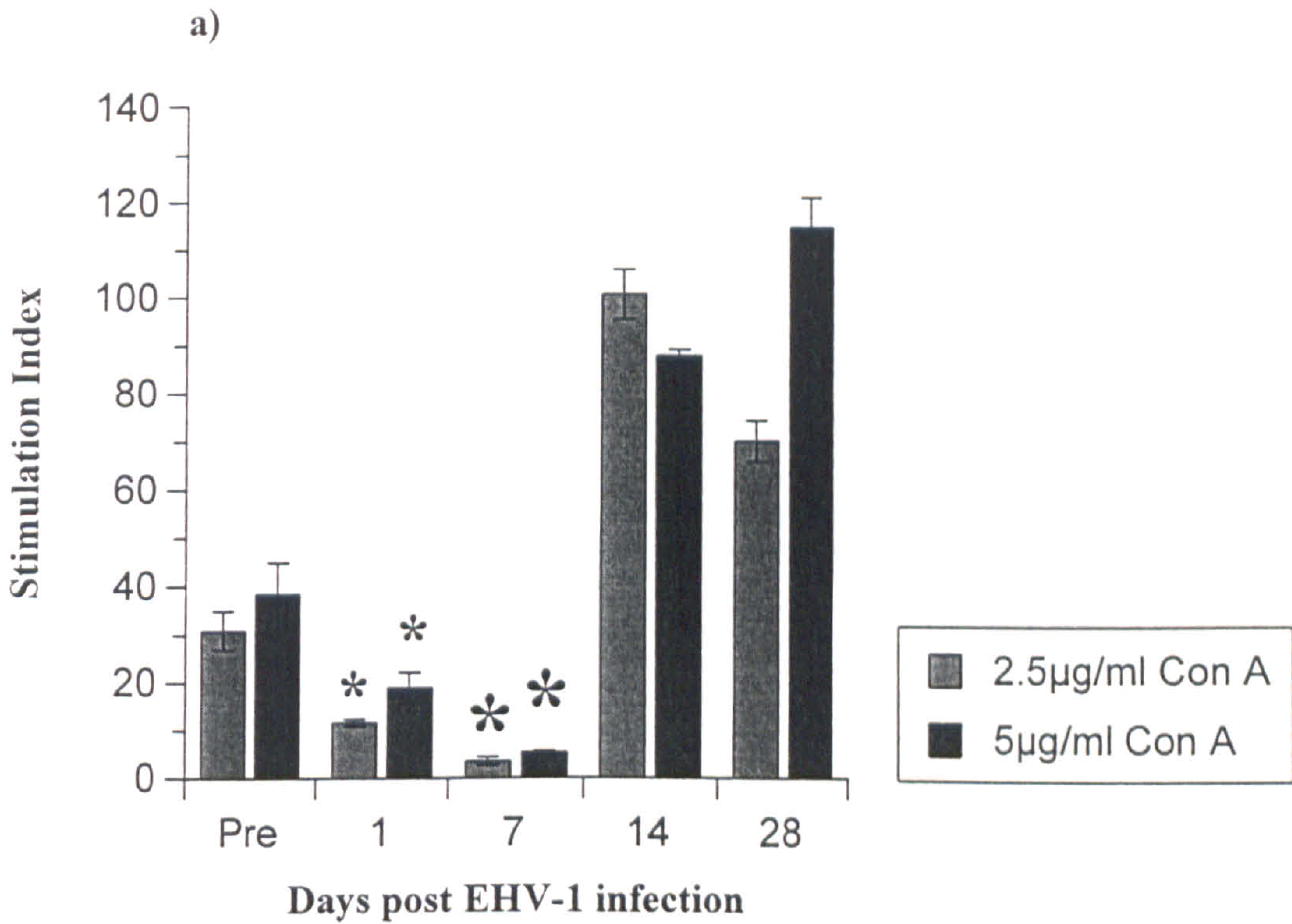
Results are mean counts min^{-1} tritiated thymidine uptake $2x10^{5}$ $cells^{-1}$.

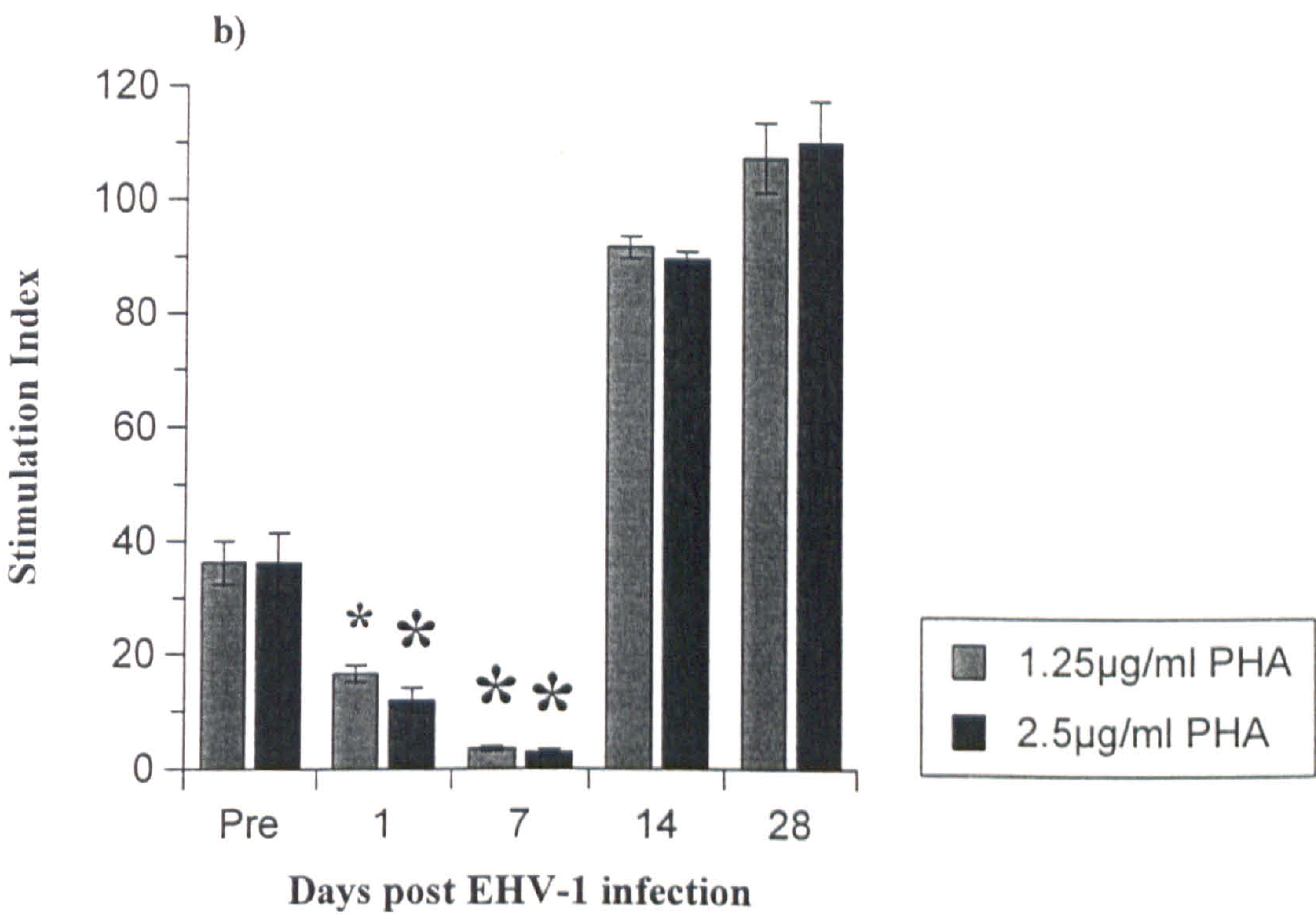
Day post-infection	Mean CPM				
	with PHA (μg/ml)		with Con A (μg/ml)		
	1.25	2.5	2.5	5.0	Bkg
Horse #219					
Pre-infection	1655	1323	2708	3924	**123**
7	5482	4384	7152	11764	**225**
14	6267	4303	10513	6266	**1841**
21	4919	3531	5407	4192	**519**
Horse #302					
Pre-infection	3471	2328	2450	4243	**79**
7	7604	8288	7934	14608	**228**
14	4444	3151	8652	5568	**556**
21	3535	2721	5407	5052	**175**

BKG = Background cpm without mitogen.
CPM = Counts per minute.

Figure 4.6. Mitogen-induced proliferation of PBMC from mare #24 after EHV-1 infection.

a) Con A induced proliferation
b) PHA induced proliferation



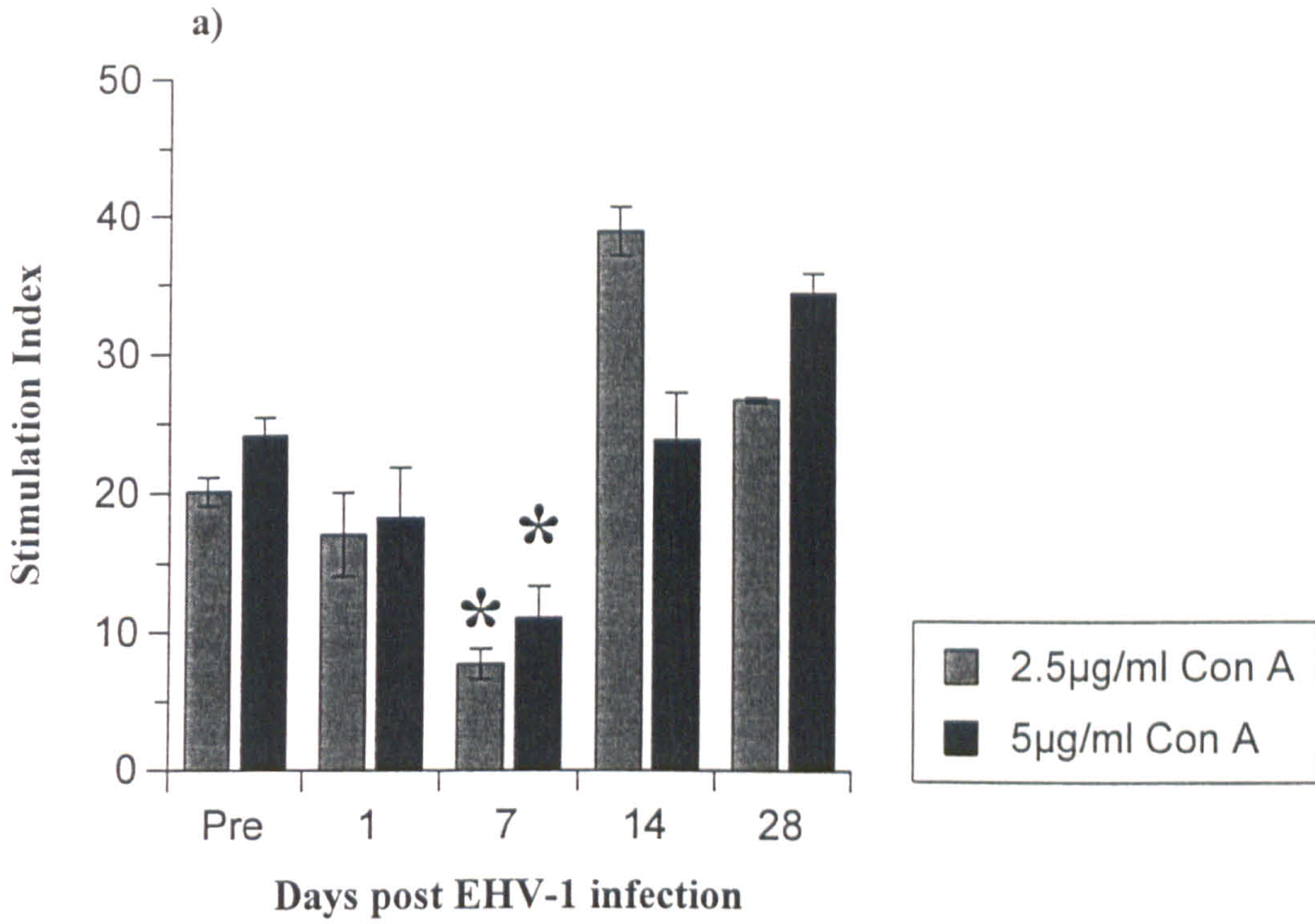


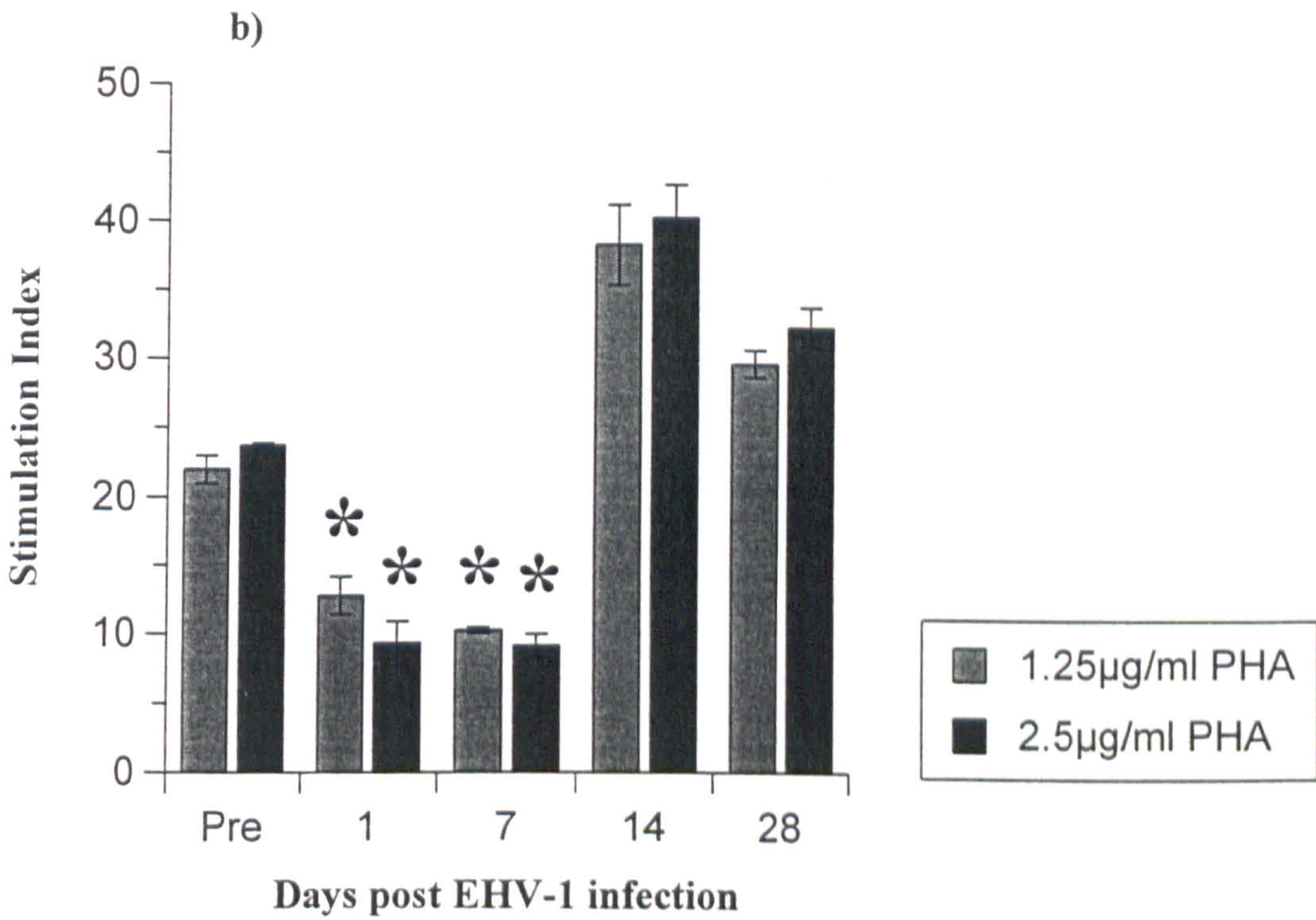
* = a significant drop in SI $p<0.001$.

* = a significant drop in SI $p<0.01$.

Figure 4.7. Mitogen-induced proliferation of PBMC from mare #26 after EHV-1 infection.

a) Con A induced proliferation
b) PHA induced proliferation





* = a significant drop in SI $p<0.001$.

Table 4.4. Proliferation of PBMC from mares #24 and #26 in response to mitogens before and after infection with EHV-1.

Results are mean counts min^{-1} tritiated thymidine uptake $2x10^5$ $cells^{-1}$.

Day post-infection	Mean CPM with PHA (μg/ml)		with Con A (μg/ml)		
	1.25	2.5	2.5	5.0	**Bkg**
Horse #24					
Pre-infection	29227	29219	29600	36842	**886**
1	891	643	626	1020	**54**
7	1171	919	1149	1793	**331**
14	38974	38085	42893	37497	**427**
28	28770	30815	18830	30815	**269**
Horse #26					
Pre-infection	26083	28118	18866	22611	**1062**
1	603	435	800	857	**47**
7	917	819	689	988	**90**
14	43540	45813	44333	27137	**1143**
28	14078	15405	12721	16373	**478**

BKG = Background cpm without mitogen

The virus-specific proliferative responses of PBMC from mares #219 and #302 to iEHV-1 are shown in Figure 4.8. Mare #219 had a pre-infection SI of 23.6 which fell to 3.5 on day 7, 2.1 on day 14 and 1.0 on day 21 post-infection. A rise in SI occurred 28 days post-infection. The reduction in SI on day 7 post-infection was reflected in a reduction in the mean cpm. The subsequent reductions in SI were partly due to increases in background proliferation (data not shown). Mare #302 did not respond significantly to iEHV-1 up to day 21 after infection, the SI values being 2.6 pre-infection, 0.9 on day 7, 0.9 on day 14 and 1.7 on day 21 post-infection. This lack of response was reflected when the mean cpm was analysed. Lymphoproliferative responses of whole PBMC from mares #24 and #26 to iEHV-1 were not analysed.

4.3.4. Antigen-specific proliferative LDA

The precursor frequencies of cells capable of proliferating to iEHV-1 in PBMC from all four mares before and 4-5 weeks after infection are shown in Table 4.5. The design of the proliferative LDA assay and the stringency afforded to the analyses are summarised in Appendix B. Examples of the experimental design, treatment of data and analyses of results in order to calculate the precursor frequency values given in Table 4.5 are shown in Appendix C.

Figure 4.8. EHV-1 induced proliferation of PBMC from mares #219 and #302 after EHV-1 infection.

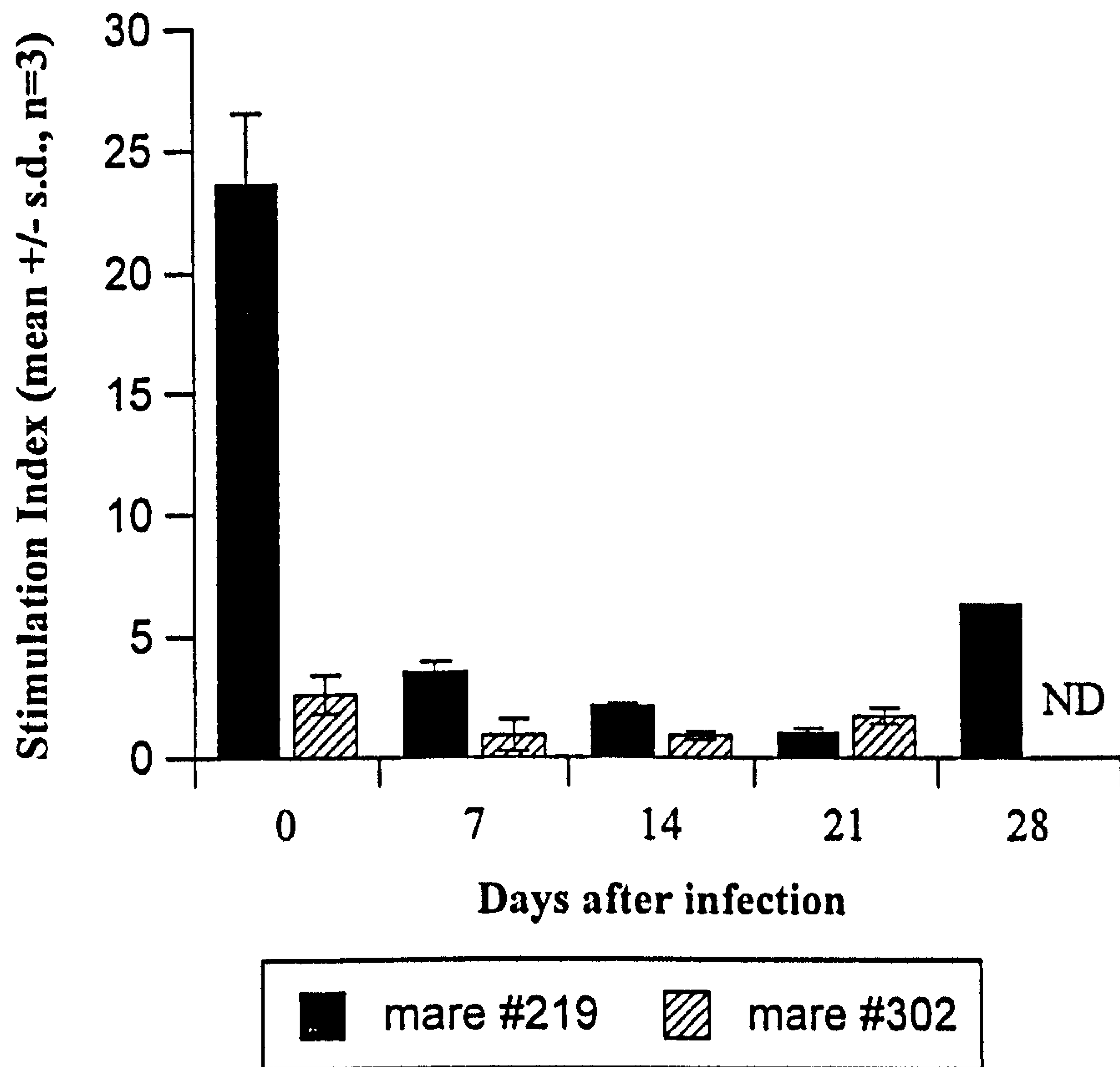

Stimulation Index (mean +/- s.d., n=3)
30
25
20
15
10
5
0
ND
0
7
14
21
28
Days after infection
mare #219
mare #302

Table 4.5. Precursor frequencies of EHV-1-specific T cells in the peripheral blood of mares before and after infection with EHV-1/Ab4/13 as measured by limiting dilution analysis of antigen-specific proliferative responses.

Reciprocal Frequencies of EHV-1 specific T cell precursors

Mare No.	Pre-infection	4-5 weeks post-infection
#24	13,889	10,309
	(10,989 to 18,868)	(8,130 to 14,286)
#26	8,547	2,532
	(6,369 to 12,987)	(1,805 to 4,255)
#219	22,222	4,651
	(16,667 to 33,333)	(3,788 to 6,024)
#302	<250,000	2,387
	(undetectable)	(1,946 to 3,096)

95% confidence limits in parenthesis.

N.B. Refer to the addendum located inside the back cover for further information concerning this data.

The frequency of precursor cells capable of proliferating to iEHV-1 before infection in PBMC from #219 was 1/22,222. This increased to 1/4,651 5 weeks after infection. The frequency of precursors in PBMC from mare #302 before infection was less than 1/250,000 (undetectable) and 5 weeks after infection was 1/2,387.

The frequencies of precursor cells in PBMC from mares #24 and #26 before infection were higher than those in cells from mares #219 and #302 (Table 4.5). This was unexpected because mares #24 and #26 had not experienced experimental infections within the previous 2 years, unlike mares #219 and #302. The pre-infection frequencies were 1/13,889 in mare #24 and 1/8,547 in mare #26. The frequencies of precursor cells 4 weeks after infection were 1/10,309 in mare #24 and 1/2,532 in mare #26.

The presence of relatively high frequencies of precursor cells capable of proliferating to iEHV-1 in PBMC from pregnant animals which became infected and subsequently aborted suggests that antigen-specific T cell responses are not indicative of protective immunity from EHV-1 disease. This is supported by the observation that the frequencies of precursor cells in the multiply infected mares WERE LOWER prior to challenge even though THEY were protected from abortion.

4.4. Discussion

The results have shown that live EHV-1 strain Ab4 suppressed the *in vitro* immune functions of equine lymphocytes. The mechanism of this suppression is unclear, although from the experiments showing the infection of equine blast cells presented in chapter 2, it is likely that the *in vitro* suppression was due to infection of stimulated lymphocytes by live EHV-1. It is clear that inactivated virus antigens only should be used for any *in vitro* study of antigen specific CMI based on proliferative responses of equine PBMC.

The clinical and virological data from the infection of ponies with EHV-1 strain Ab4/13 illustrated the importance of the collection of complete data sets for analysis. For example, although there was no obvious clear cut differences in the duration of the virus isolation from nasal swab extracts between the multiply infected (#219 and #302) and the initially infected (#24 and #26) mares, there was a difference in the total amount of excreted infectious EHV-1 when nasal swab extracts were titrated (see Table 4.2a & 4.2b). The total amounts of virus measured in nasal swab extracts from mares #219 and #302 after initial infection (Table 4.1b) indicated that this parameter was not a reliable indicator of the outcome of infection as mare #219 aborted despite excreting 100 times less virus over a 2 day shorter time period than mare #302 which produced a healthy foal. McCulloch *et. al.* (1993) carried out an exhaustive study of the changes in circulating leukocyte populations after EHV-1 infection, but did not report any data on nasal

excretion of virus or cell associated viraemia. The subsequent discussion of the inherent variability in the phenotype of cells in the blood after EHV-1 infection, therefore, took no account of the differences in the infection histories of the (previously infected) outbred horses in the study and thus missed the opportunity to correlate these changes with specific clinical outcome.

The clinical differences between the initially infected (#24 and #26) and the multiply infected (#219 and #302) mares in this study were clear. Both of the initially infected mares aborted whereas both of the multiply infected mares did not.

The responses of equine PBMC to mitogens have been studied by a number of investigators. Dutta *et. al.* (1980) showed that, after an initial increase in responsiveness to PHA, PWM and Con A, PBMC from infected mares became refractory to mitogenic stimulation. This suppression reached its lowest level 10 days after infection and recovered to near pre-infection levels by 14 days after infection. This initial increase in mitogen responsiveness prior to suppression was also reported by Bumgardner *et. al.* (1982). Hannant *et. al.* (1991) demonstrated that, after EHV-1 (strain V592) infection of young ponies, a relatively long-term suppression of the *in vitro* PHA response of PBMC ensued. This long term suppression did not occur in the present study. Instead, there was no suppression of the response to PHA or Con A in the two multiply infected animals, whilst a transient suppression followed by a significant enhancement of the response occurred in the two initial infection animals.

The reasons for these changes in responsiveness to mitogens after EHV-1 infection are unclear. During the non-responsive period, the only virus isolated, by co-cultivation of samples on a permissive cell line, was from the blood. The viraemia which occurs after EHV-1 infection typically lasts for up to 21 days (Mumford *et. al.*, 1994) after infection as does the period of *in vitro* non-responsiveness using EHV-1 strain Ab4 (D. Hannant, AHT, Newmarket, personal communication). The observation of a temporal correlation between these two phenomena suggests that they are causally related. The data presented in Figures 4.6 and 4.7 support this speculation, as the periods of viraemia in the two mares span the periods of the most significant decreases in mitogen responsiveness (#24 viraemic between days 3 and 11 after infection, #26 viraemic between days 5 and 11 after infection).

The data presented in this chapter show that live EHV-1 interferes with the *in vitro* responsiveness of equine PBMC to mitogens (see figures 4.1 & 4.2). This has also been reported for bovine herpesvirus type 1 (BHV-1). Moreover, live BHV-1 is also able to reduce the proliferation of lymphoblasts to interleukin 2 (IL2, Carter *et. al.*, 1989). Allen *et. al.* (1995) showed that EHV-1 was able to replicate in Pokeweed mitogen (PWM) induced blast cells but not in unstimulated equine PBMC (see also Thomson & Mumford, 1977). Given that EHV-1 has not been shown to grow to any extent in equine monocytes, polymorphonuclear leukocytes or B cells, it may be that *in vivo* activation of PBMC could facilitate the cell to cell spread of virus for a period of up to 21 days, at which time virus-specific cell-mediated immune responses clears the viraemia. In primed

animals, T cell memory is enhanced, therefore cell associated viraemia is cleared quicker than in naive animals.

Evidence exists for the *in vivo* activation of equine PBMC after EHV-1 infection. Thomson *et. al.*, (1976b) reported the spontaneous proliferation of equine PBMC *in vitro* after EHV-1 infection, whilst McCulloch *et. al.*, (1993) reported the presence of blast cells in the blood of horses after infection, detected by analysis with a fluorescence activated cell sorter (FACS). These blast cells were readily detectable 4 days after infection and observed for a further period of about 10 days. It was not reported whether these blast cells were T cells, or whether they were permissive to EHV-1 infection, although they are obvious candidates for further study. If these blast cells are the source of viraemia, control by T cell-specific mechanisms has been suggested by the finding that treatment of SPF foals with cyclosporin A (CyA) during an EHV-1 challenge prevented clearance of viraemia (Slater *et. al.*, 1994). Viraemia was only cleared from these foals when CyA treatment was discontinued, suggesting a T cell-mediated clearance mechanism.

The inherent variability of antigen-specific proliferative assays, after infection with EHV-1, was also demonstrated by Allen *et. al.* (1995) who showed that infection of six horses with EHV-1 strain A183 resulted in the production of antibody (as measured by ELISA) and antigen-specific *in vitro* proliferation of PBMC. Both of these immune parameters were variable between horses with the peak ELISA antibody titre varying between

approximately 1/4,000 and 1/12,000 and the peak antigen-specific proliferation SI varying between approximately 8 and 80. High ELISA antibody titre did not seem to correlate with high SI.

The proliferative LDA described in this chapter was used primarily as a technique to determine whether the frequency of precursor T cells detected before challenge with EHV-1 correlated with immune status. The precursor frequency of antigen-specific T cells increased in 3 out of 4 horses after infection (Table 4.6). However, as the pre-infection frequencies of responding cells in the two initially infected mares were higher than those in the two multiply infected mares, no correlation between frequency and outcome of infection seemed to exist. The lack of antigen-specific T cell responses in bulk culture seen after infection was reflected in the proliferative LDA results. For example, the complete lack of any antigen-specific proliferative response in PBMC of mare #302 up to 21 days after infection was mirrored by the very low frequencies of proliferative cells in this animal before infection.

Further work needs to be done to clarify the events which occur in the acute phase after EHV-1 infection. This work needs to include experiments to define the cell types involved in the establishment and control of viraemia, as this seems to be of central importance to the progression of disease. Finally, more relevant immune parameters must be identified in order to predict protection against and recovery from EHV-1.

5. Measurement of the frequency of CTLp using limiting dilution analysis

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5.1. Introduction

The genetic (Studdert *et. al.*, 1981), serologic (Horner, 1981) and pathogenic (Edington, 1990) relationships between EHV-1 and EHV-4 have been well studied. However, there is no information on the potential for these viruses to stimulate CTL precursors (CTLp) to develop into effector CTL *in vitro* after infection.

In chapter 3 it was shown that cross-reactive CTL activity could be induced in bulk cultures of PBMC of horses after infection with EHV-1/EHV-4. These bulk culture CTL assays had the capacity to distinguish between naive and previously infected horses (see chapter 3) but were less reliable when comparing CTL activities between the latter. In contrast, there is evidence that the measurement of the CTL precursor frequency may provide a more valuable indicator of effective CMI responses to virus infections (Doherty *et. al.*, 1994. Klein *et. al.*, 1995). The experiments described in this chapter were designed to determine the frequencies of CTLp present in the blood of horses before and after infection with EHV-1 or EHV-4 and to assess the contribution of CTL to recovery from disease. Because the animals which were available for this study were adult horses, they were likely to have experienced previous infections with both viruses. In order to estimate precursor frequencies of both EHV-1 and EHV-4 CTLp from previously infected animals, a CTL limiting dilution analysis (LDA) assay was developed. This chapter describes the development and application of this assay to the measurement of CTL precursor frequency in peripheral blood.

5.2. Material and Methods

5.2.1. Animals and infection

Two infection experiments were carried out. In the first, 2 multiply previously infected adult Welsh mountain pony mares were re-stimulated with $1 \times 10^{6.6}$ $TCID_{50}$ EHV-1 strain Ab4/8 by exposure to a nebulised aerosol as described (Mumford *et. al.*, 1994). In the second experiment, 4 Welsh mountain ponies were infected with EHV-4 strain V1778 ($10^{5.6}$ $TCID_{50}$ total virus nebulised) as described (Mumford *et. al.*, 1994). Subsequently, 0.5ml of the same virus was introduced into each nostril of each pony using a flexible plastic pipette. This strain of EHV-4 had not been extensively grown in tissue culture (used at 2nd passage in equine embryonic lung, EEL cells).

5.2.2. Sampling, serology and virus isolation

After EHV-1 and EHV-4 infection, the ponies were sampled regularly for the determination of serum complement fixing (CF) antibody (Thomson *et. al.*, 1976a) and virus isolation from nasal swabs. Heparinised blood samples were taken on days 2, 4 and 6 after infection in order to detect any viraemia due to infectious virus (EHV-1 experiment) or re-activated EHV-1 (EHV-4 experiment), they were treated as described (Mumford *et. al.*, 1987). Clinical observations including rectal temperatures were performed daily for two weeks (Mumford *et. al.*, 1994).

5.2.3. EHV specific CTL LDA split well assay

A split well LDA for CTL was developed based on that used to enumerate human cytomegalovirus specific CTLp (M. Wills, Department of Medicine, University of Cambridge, personal communication). In order to measure the precursor frequency of CTL which were virus-specific and genetically restricted, effector CTL were induced in individual wells of a microtitre plate for 7 days. The induction cultures are described in 5.2.3.1. below. After induction, the cells in each well were split into three aliquots. The resultant effector cells were assayed using ^{51}Cr-labelled autologous EHV-1 infected cells, MHC mis-matched target cells (to control for allogeneic killing) and autologous mock-infected target cells (to control for promiscuous killing).

PBMC were isolated from the ponies to be tested and from an ELA mis-matched horse. The ELA typing of all ponies used in this study was carried out by isoelectric focusing of immune-precipitated ELA class I molecules. This was kindly performed by Dr. M. Whelan, BBSRC Institute for Animal Health, Compton. All cells were suspended in AIM-V/SM + 5% autologous serum at $1x10^8$ cells ml^{-1} (see appendix A for a description of the media formulations).

5.2.3.1. Filler cells for CTL LDA

$4x10^7$ autologous PBMC were treated with Mitomycin C (Mit C, 50μg Mitomycin C in 1ml of HBSS at 37°C for 30 min, then wash extensively) and resuspended at $6x10^5$ cells ml^{-1} in AIM-V/SM + 5% autologous serum with the following additives:

a) 10% equine conditioned medium (CM) from Con A stimulated PBMC.

b) 20IUml^{-1} recombinant human IL2.

c) An optimum dilution of live EHV-1 or EHV-4. This depended on the $TCID_{50}$ of the virus preparation in use at the time (see below).

5.2.3.2. Induction of CTL under LDA conditions

24 wells of a 96 round bottomed plate were filled with 200µl of live autologous PBMC at $5x10^5$ cells ml^{-1} in AIM-V/SM + 5% autologous serum and 100µl of cells from the first set of wells was transferred using a multichannel pipette into the second 24 wells. The pipette tips were changed and 100µl of fresh medium was added to the second set of wells. This procedure was repeated until 7 sets of 24 wells (i.e. 24 wells short of 2 plates) contained 100µl of cell suspension in each well. One hundred microliters of fresh medium alone was added to the final set of 24 wells. The dilutions represented a range of cell concentrations from 50,000 cells per well to 781 cells per well. One hundred microliters of the Mit C treated filler cell suspension containing $6x10^4$ cells was then added to each well. Thus, the final set of 24 wells contained filler cells only.

The plates were then incubated at 37°C in an atmosphere of 5% CO_2 for 7 days.

5.2.3.3. Target cell preparation

The remaining PBMC in AIM-V/SM + 5% autologous serum at $1x10^8$ cells ml^{-1} were incubated in 50ml tubes with loose tops at 37°C for 5 minutes in an atmosphere of 5%

CO_2 in air. The tops were then tightened and the tubes were stored at 4°C for 4 days. After 4 days PWM blasts were produced from the stored cells as described in 3.2.3. After 48 hours in culture with PWM, the blast cells were infected and labelled with ^{51}Cr as described in 3.2.3 in order to produce three sets of targets per pony tested, i.e. autologous EHV-1 infected, mis-matched EHV-1 infected and autologous mock-infected. After 12 hours of infection/labelling the target cells were treated as described in 3.2.3.

5.2.3.4. CTL assay under LDA conditions

Each well of the induction cultures was split into three aliquots, with each new well containing 66µl from the original (one third of the original volume). Pipette tips were changed between each transfer, as each well potentially contained a different number of effector CTL compared with the others.

Each set of plates was labelled as containing either autologous, infected; mis-match, infected or autologous, mock-infected targets. 100µl ($1x10^4$) of each target cell was added to each well of the appropriate plates and the plates were then processed as for the standard CTL assays described in 3.2.3. Supernatants were harvested in strict order so as to allow comparison of CTL activities from one well against the 3 target cell types.

Specific cytotoxicity values were derived for each well from the plates containing the 3 target cell types. Cytotoxicity results derived from wells containing autologous virus-infected target cells were compared with the equivalent wells containing MHC mis-

matched virus-infected and autologous mock-infected target cells. If the CTL activity in a well containing autologous virus-infected cells had a % specific release value 10% above those of both the activity in an equivalent well containing mis-matched, infected or autologous, mock-infected targets, then it was scored as positive. Data were analysed using the LDA program described in 4.2.4 and appendix B. Examples of the raw data produced and the results obtained from this type of experiment are shown in appendix D.

5.2.3.5. Optimisation of the assay

An experiment was carried out in order to determine which components of the CTL LDA assay were necessary for the efficient maturation of CTLp to effector CTL. EHV-1 specific CTLp were assayed from a multiply infected horse using the full assay (see above), without filler cells alone, or without filler cells and CM/rhIL2.

The proliferative status of the cells in a CTL LDA assay on days 4 to 7 of induction was also determined in order to assess the contribution of proliferation to the maturation of CTLp into effector CTL. This was done by setting up culture wells (n=6) containing $5x10^4$ PBMC, varying the culture conditions and pulsing the cells in the assay for 18 hours with tritiated thymidine after 4, 5, 6 and 7 days. Harvesting and counting of the labelled cells was carried out as described in chapter 3.

5.3. Results

5.3.1. Development of a limiting dilution analysis (LDA) CTL assay

Because incubating duplicate plates for either 7 or 14 days resulted in similar frequency estimates (data not shown) the shorter, more convenient, 7 day incubation was used throughout. Also, the shorter incubation period meant that there was less chance of particularly active cultures depleting nutrients from the medium and thus affecting the frequency determination.

Table 5.1 shows the effect on the CTLp frequency determination of omitting either filler cells or CM/rhIL2 from the assay. All other components were as described in section 5.2.3 except that the cells used in this experiment were recovered from cryopreservation. When the full assay was performed the CTLp frequency of EHV-1 specific genetically restricted CTL in PBMC from this mare (#302 sampled before experimental infection) was 1/19,231. When fresh cells from the same bleed were used in a similar assay the CTLp frequency was 1/13,158. These values were not significantly different (using the non-overlap of 95% CIs as an indication of a clear significant difference in CTLp frequency between two assays, Fischer *et. al.*, 1991) which showed that cryopreservation of PBMC did not adversely effect the CTLp frequency. When filler cells were omitted from the assay the CTLp frequency was 1/32,258 and when filler cells, CM and rhIL2 were omitted (*i.e.* the assay contained effector cells and antigen only) the CTLp

Table 5.1. The effect on CTLp frequency determination of omitting either filler cells or conditioned medium/rhIL2 from the assay.

CTL assay	Reciprocal pCTL frequency	95% CI
Full assay	19,231	14,706 to 27,027
No filler cells	32,258	25,000 to 45,456
No fillers/No CM/IL2	58,824	43,478 to 90,909

frequency was 1/58,824. These results show that omitting any component of the CTL LDA assay had the effect of reducing the CTLp frequency. Although there were overlaps in the 95% CIs from these estimates, they were slight and the differences between the estimates were large.

There was significant proliferation within the culture wells over the 7 day period of the assay. However, Table 5.2 shows that omitting these components of the CTL LDA assay had the effect of reducing lymphocyte proliferation within the culture wells. There was an increase in proliferation when effector cells were cultured alone over a 7 day period, and in the assays examined to date this resulted in a small, but significant effector CTL production. There was also evidence for an increase in the thymidine uptake in the wells containing filler cells and antigen only (Table 5.2) between day 4 and 7 in culture. However, this never resulted in the generation of effector CTL.

The determination of the CTLp frequency of cells induced from PBMC with EHV-1 and EHV-4 is shown in Table 5.3. The frequency of EHV-1 strain Ab4/13 specific CTLp in PBMC from an immune mare 193 days after an experimental infection with homologous virus was 1/19,603 (95% CI 15,152 to 27,278). The CTLp frequency of EHV-4 strain MD cross-reactive CTL in the same PBMC population was 1/16,667 (95% CI 12,658 to 23,810). As the 95% CI values overlapped extensively these frequencies were not considered significantly different.

Table 5.2. Proliferative response of cells in a CTL LDA assay and the effect on proliferation of omitting components of the assay.

Assay treatment	Day of induction 4	5	6	7
Full assay	**1238**	**4574**	**16627**	**35643**
	±228	±1084	±5631	±5249
Antigen only	**1089**	**3998**	**6877**	**10697**
	±746	±1313	±3451	±5359
Effectors	**513**	**1519**	**4069**	**6283**
	±177	±764	±2406	±3647
Fillers	**102**	**89**	**855**	**1292**
	±11	±29	±941	±2497

Table 5.3. The precursor frequency of CTL induced with EHV-1 and EHV-4 in PBMC from an immune (i.e. multiple previous exposures to EHV-1) mare.

CTL inducing antigen	Reciprocal pCTL frequency	95% CI
EHV-1 strain Ab4/13	19,608	15,152 to 27,278
EHV-4 strain MD	16,667	12,658 to 23,810

5.3.2. CTLp before and after challenge with EHV-1

The CTLp of EHV-1 specific CTL in PBMC from two multiply infected mares (#302 and #219) were measured before and after infection with EHV-1. Experimental infection of these animals did not result in pyrexia (a body temperature of above 38.9°C), or nasal shedding of infectious EHV-1. Only one day of viraemia was detected in one of the 2 mares (#219 day 8 post-infection). Furthermore, there were no sero-conversions, as detected by the CF test, up to 35 days post-infection, although there were low levels of pre-existing CF antibody before infection. The clinical and virological measurements were consistent with the animals having a degree of immunity to EHV-1 induced by previous infection.

The frequencies of EHV-1 specific CTLp in PBMC from both infected mares and from one non-infected control mare (which had received no previous experimental infections) before, 17 days after and 41 days after infection are shown in Table 5.4. Pre-infection CTLp frequencies were high in both previously infected mares (1/13,158 in #302 and 1/17,857 in #219). The CTLp frequency in PBMC from the control mare was <1/150,000. This notation was used as the 95% CIs from estimates of frequencies less than 150,000 were so large that the accuracy of the estimate could not be guaranteed. These frequencies reflect the mares previous experience of EHV-1 and/or EHV-4 infection. The multiply previously infected mares had high frequencies of CTLp whilst the non-previously infected mare had a very low CTLp frequency. After infection, the CTLp frequency in PBMC from mare #302 did not alter significantly, whilst the

Table 5.4. Frequency of CTLp in PBMC from two infected mares and one non-infected control mare (which had received no previous experimental infections) before, 17 days after and 41 days after infection with EHV-1.

	Reciprocal pCTL frequency		
Horse number	Before infection	17 days after infection	41 days after infection
#302	13,158	14,085	13,514
#219	17,857	52,632	11,764
#310	<150,000	<150,000	Not tested

After challenge of horses #302 and #219 with EHV-1, infection could only be demonstrated (by cell associated viraemia on one day only) in #219.

frequency in PBMC from mare #219 decreased to 1/52,632. There was no significant change in the CTLp frequency in the uninfected control mare after this period. The decrease in CTLp in mare #219 may have been due to the transient viraemia detected at day 8 post-infection in this horse. CTLp frequencies were measured again 41 days after infection in order to determine whether the decrease in CTLp from mare #219 was transient or sustained. The frequency of CTLp in PBMC from mare #302 remained at 1/13,514, whilst the frequency in PBMC from mare #219 increased significantly to 1/11,764. The transient decrease in CTLp in PBMC from mare #219 warrants further investigation.

5.3.3. EHV-4 challenge experiment

An experimental EHV-4 infection of 4 young animals was carried out in order to determine the importance of CTL in protection from EHV-4 infection and also to assess the potential of bulk culture and LDA CTL assays to distinguish between uninfected and infected animals.

All animals were screened for CF antibody two months before infection, at which time 2 animals (#1 and #2) were seronegative whilst 2 (#3 and #4) showed serological evidence of recent previous infection (see below).

	Complement fixing antibody titre	
Pony number	EHV-1 Ab4	EHV-4 MD
1	-	-
2	-	-
3	40	5
4	160	40

Table 5.5. CF titres of 4 ponies two months before an EHV-4 challenge experiment.

CF antibody titres on the day of infection (day 0) showed that ponies #1, #2 and #3 had no antibody to either EHV-1 or EHV-4 whilst pony #4 showed a residual CF titre of 40 to EHV-1 and 10 to EHV-4. The post-infection CF antibody titres can be seen in Table 5.6. It is clear that ponies #1, #2 and #3 sero-converted to both EHV-1 and EHV-4 whilst pony #4 did not.

The body temperature of each pony was taken daily for five days after infection. The only signs of pyrexia (a body temperature of above 38.9°C) were in ponies #1 and #2 on day 2 after infection. Nasal excretion of live virus was observed on one occasion only after infection, in a nasal swab extract taken from pony #1 (a titre of $10^{3.1}$ $TCID_{50}ml^{-1}$ measured on day 4). No EHV-1 or EHV-4 was isolated from heparinised blood samples, although EHV-2 (confirmed by immunofluorescence) was isolated from samples taken from ponies #3 and #4 on day 6 after infection.

Table 5.6. Complement fixing antibody response of EHV-4 infected animals before and after infection.

Horse # / Days after inf		Antigen used in CFT	
		EHV-1 Ab4	EHV-4 MD
#1	0	-	-
	4	-	-
	7	80	160
	14	80	160
#2	0	20	-
	4	-	-
	7	20	20
	14	80	80
#3	0	-	-
	4	10	-
	7	40	40
	14	40	20
#4	0	40	10
	4	20	10
	7	20	10
	14	10	10

A sero-conversion is considered to be a four fold increase in CF titre, *e.g.* 1/20 to 1/80.

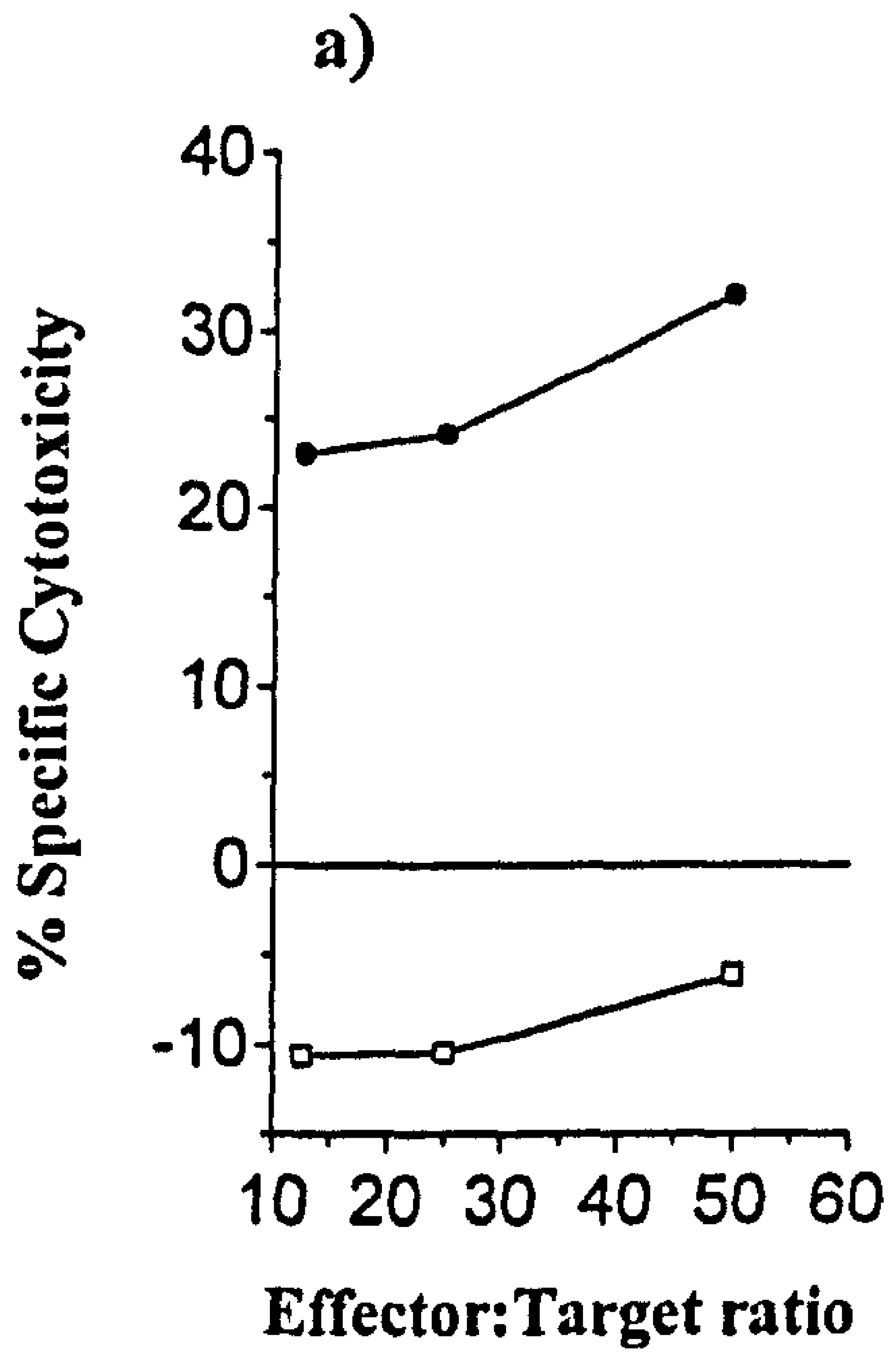
The above evidence suggests that ponies #1 and #2 had not experienced recent infection with either EHV-1 or EHV-4 and were susceptible to infection with EHV-4 (pyrexic response in both and nasal excretion of virus in one pony). Ponies #3 and #4 however, had evidence of recent exposure to either EHV-1 or EHV-4 and were essentially protected from EHV-4 infection although antibody levels were boosted in pony #3.

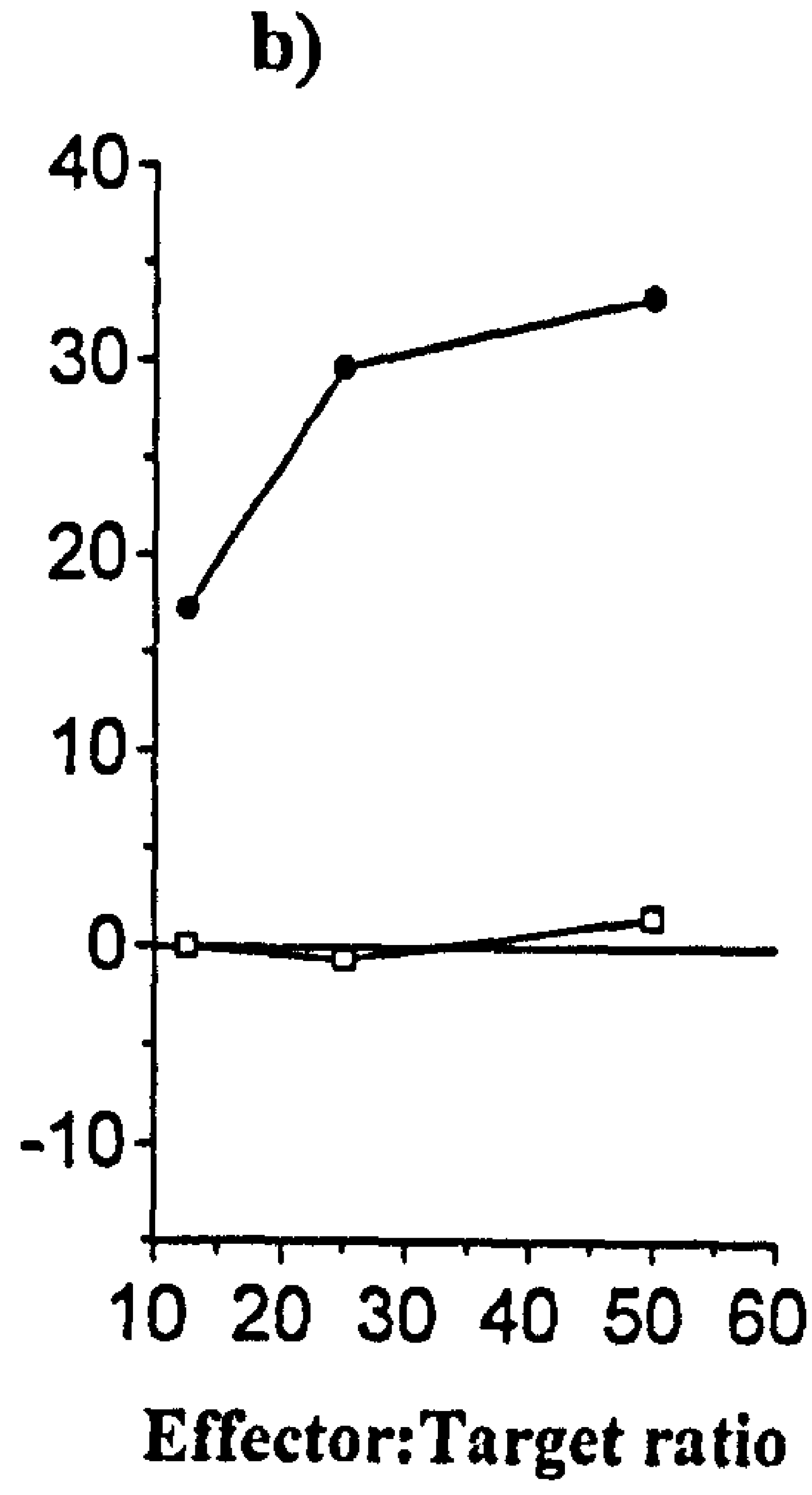
Bulk culture CTL assay results can be seen in Figures 5.1i and 5.1ii. These Figures show pre-infection (a, c, e and g) and 28 days post-infection (b, d, f and h) CTL activity in PBMC from each pony stimulated with EHV-4 strain MD (solid circles) and mock antigen (open squares). It is clear from these results that before infection, all 4 ponies showed CTL activity when PBMC were stimulated with EHV-4. Despite minor variations, post-infection CTL activities stimulated by MD were similar to pre-infection activities.

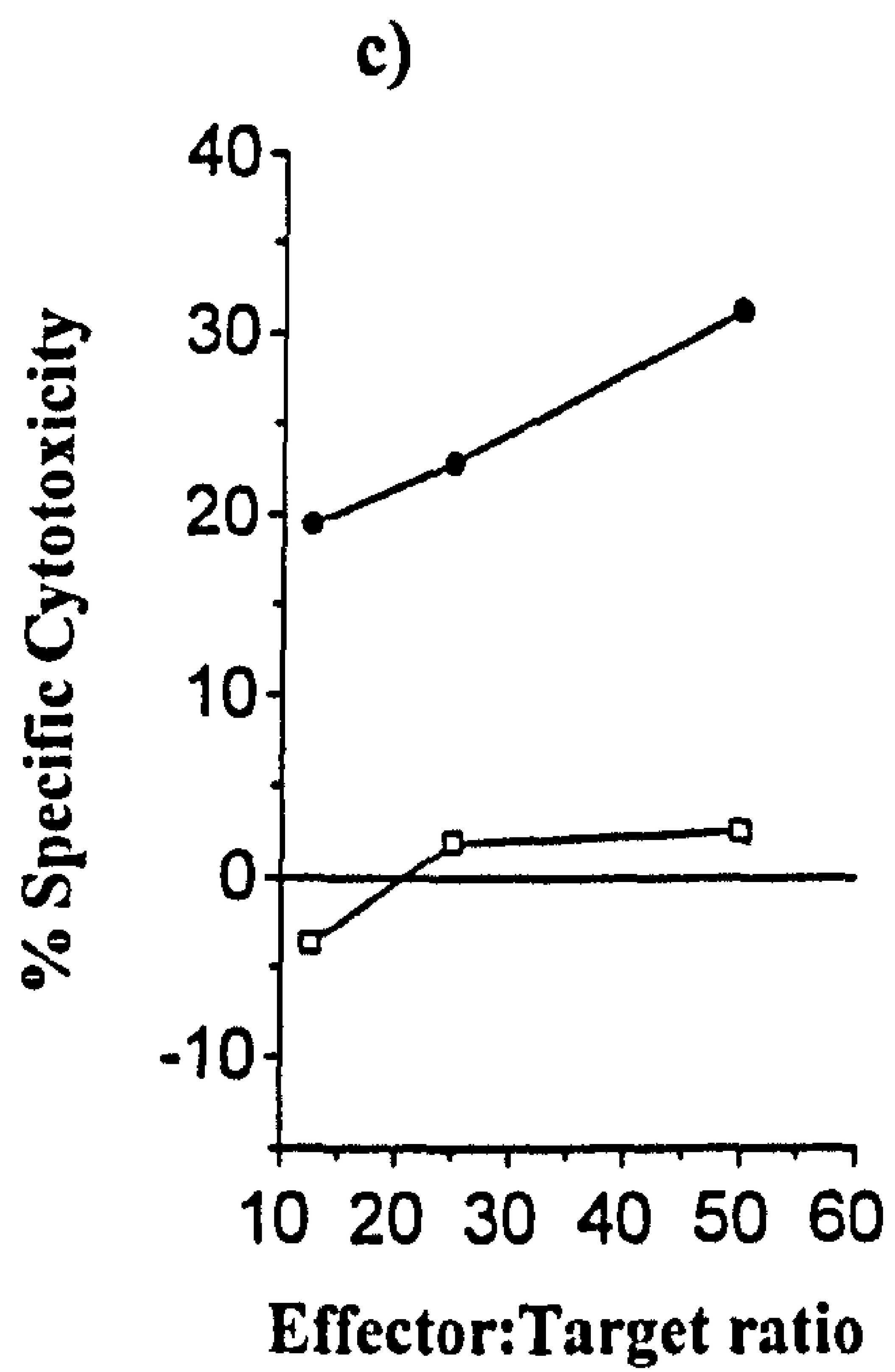
The frequency of EHV-4 cross-reactive CTLp in PBMC from the four animals before and 10 days after infection can be seen in Table 5.7. Before infection only pony #1 showed a measurable frequency (1/125,000). The frequencies of CTLp in PBMC from the other three ponies were <1/150,000. After infection, the frequencies of CTLp in PBMC from ponies #1 and #2 increased significantly to 1/15,625 (95% CI 1/11,765 to 1/23,810) and 1/33,333 (95% CI 1/22,222 to 1/62,500) respectively. The post-infection CTLp frequencies in PBMC from ponies #3 and #4 were <150,000 and 1/111,111 (95% CI 1/76,923 to 1/200,000) respectively.

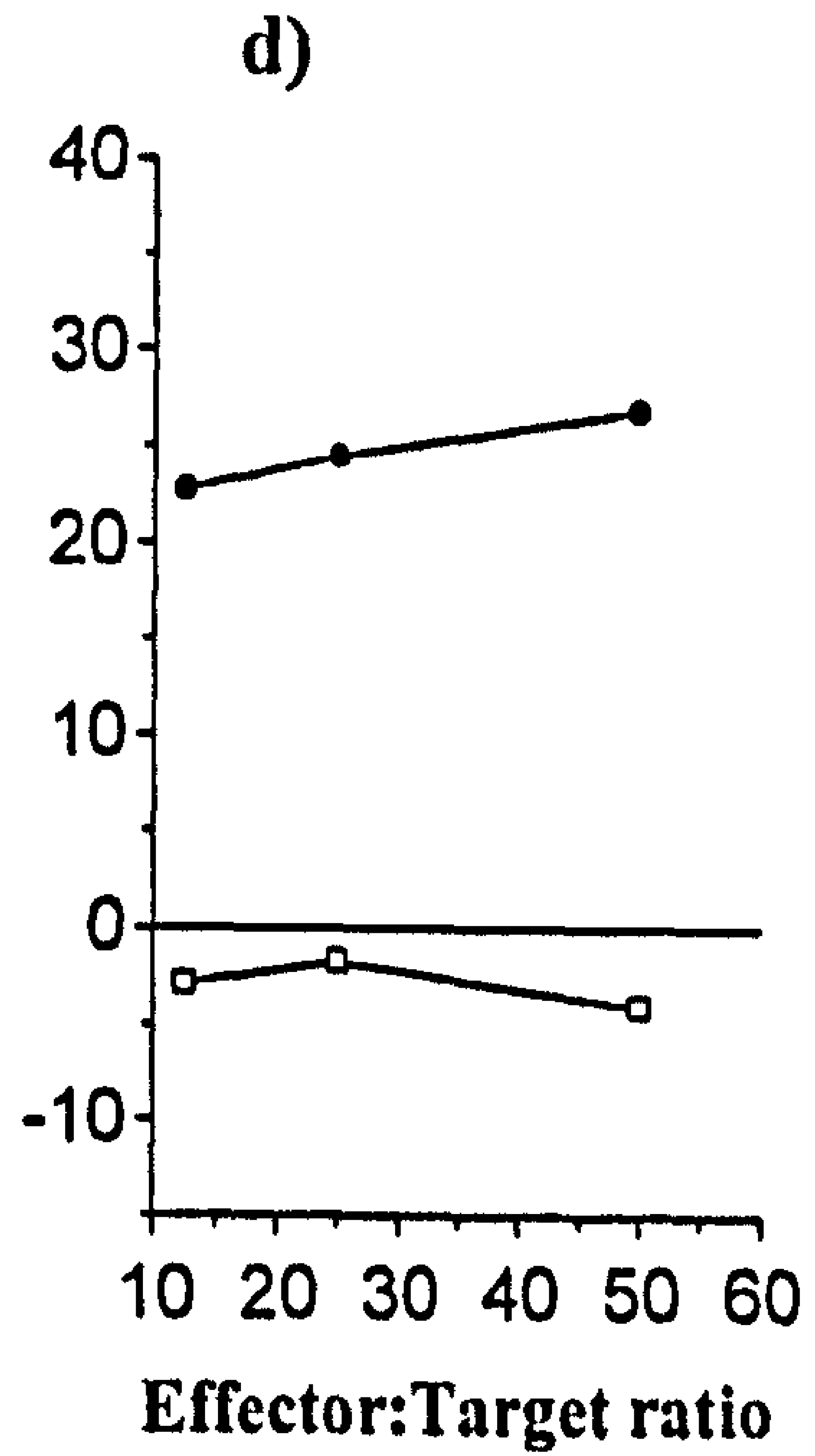
Figure 5.1i. Bulk culture CTL activity in PBMC from 4 ponies before and 28 days after infection with EHV-4.

a) #1 before infection
b) #1 after infection
c) #2 before infection
d) #2 after infection







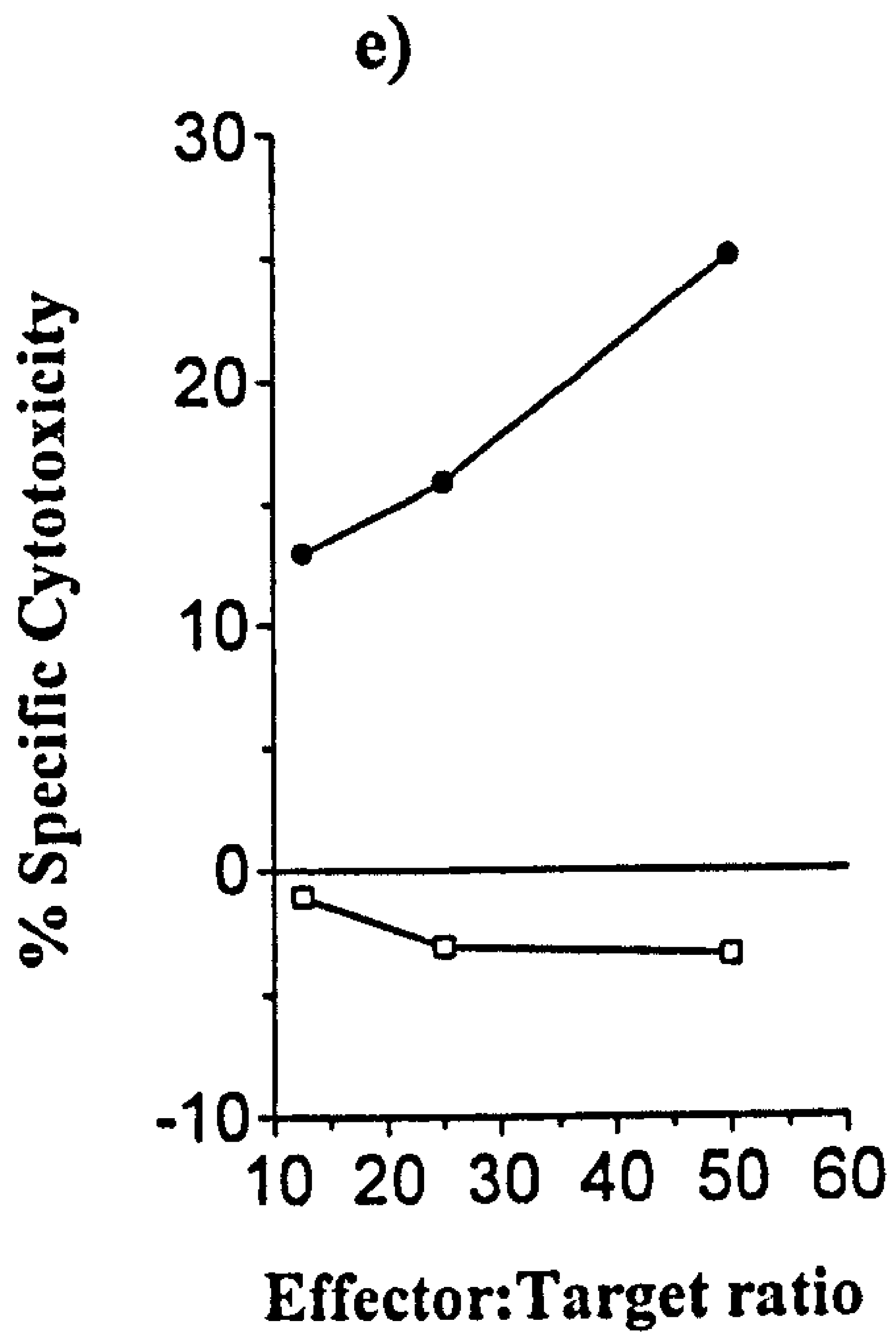


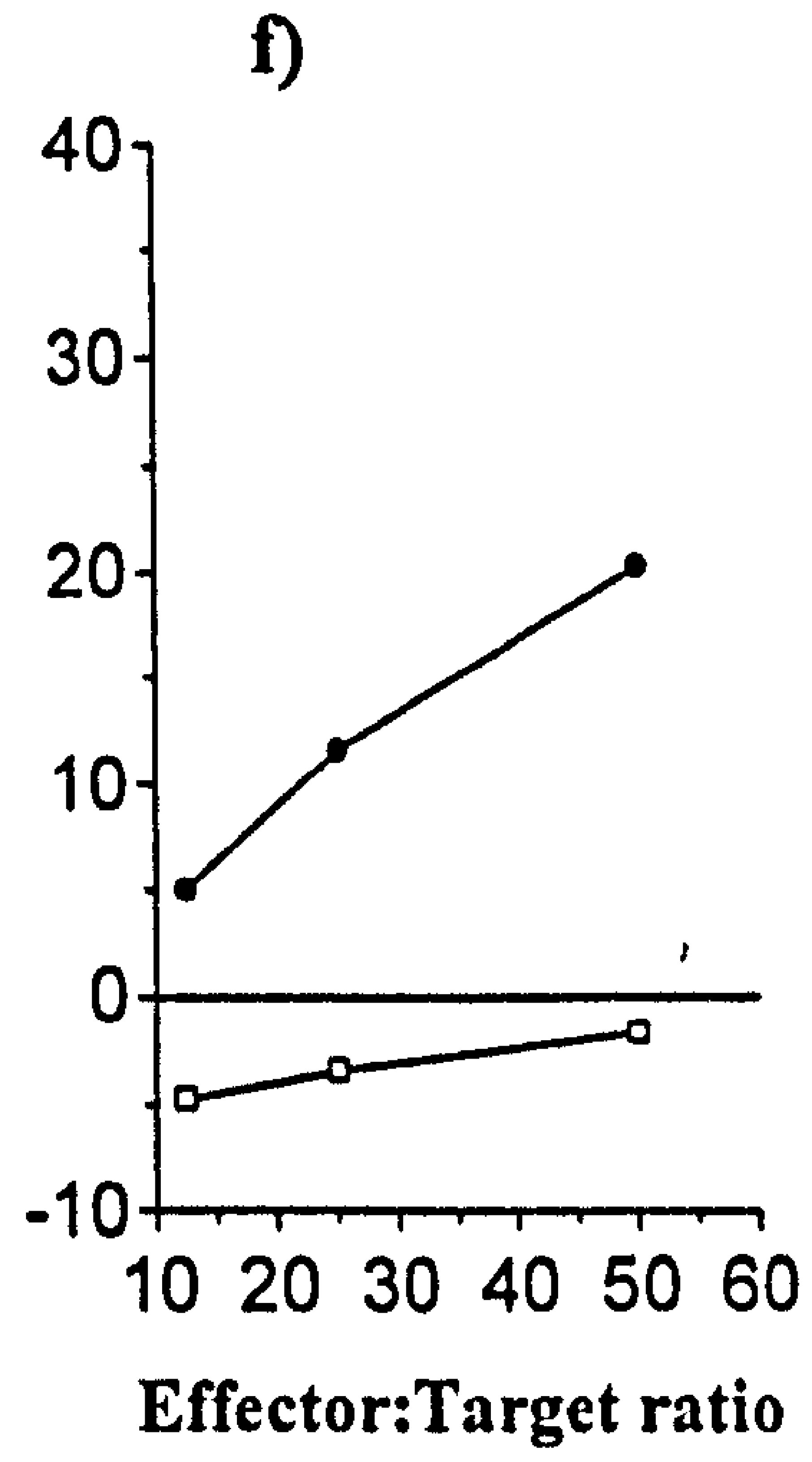
Inducing Antigen:

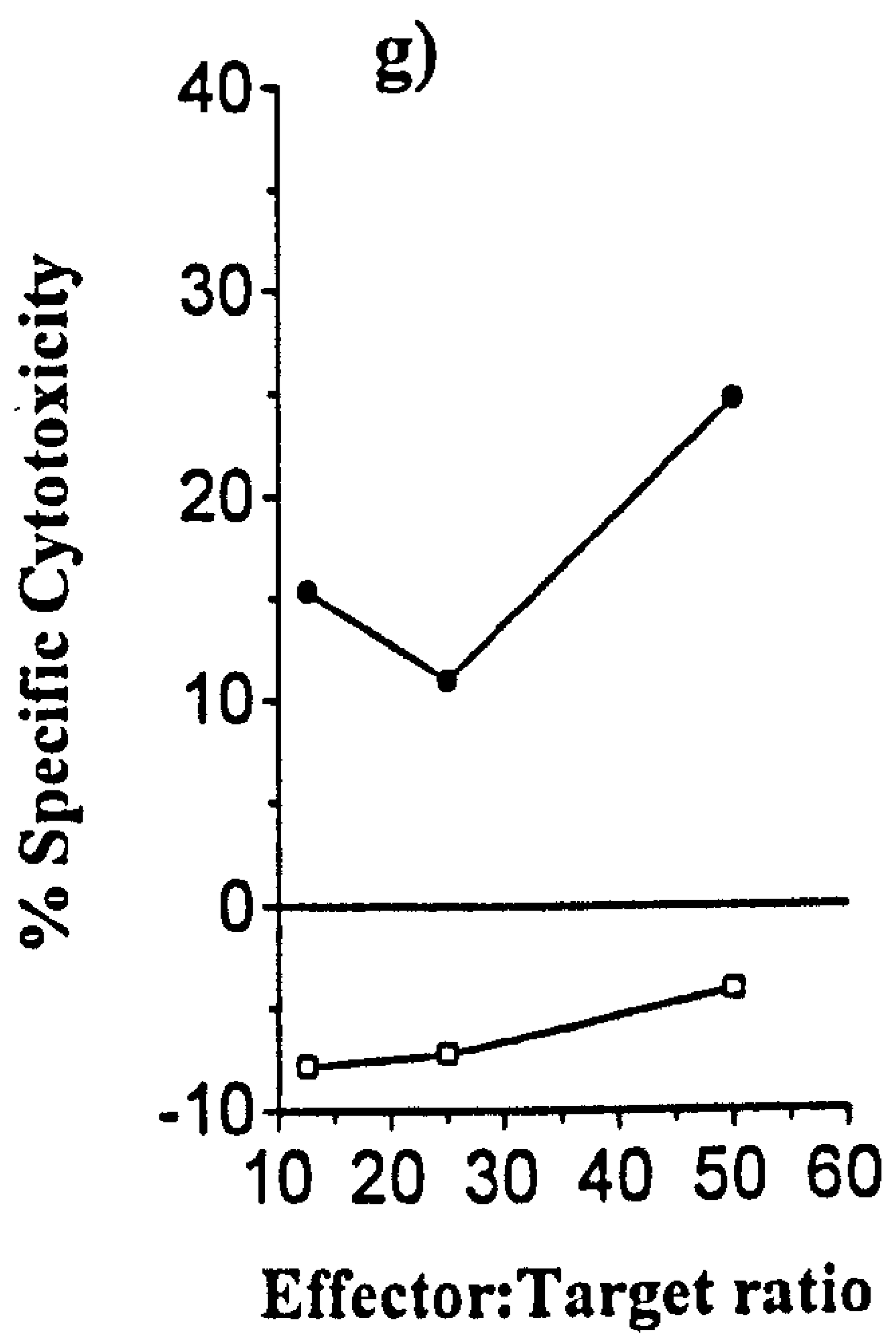
◆ EHV-4 induced	▫ Mock induced

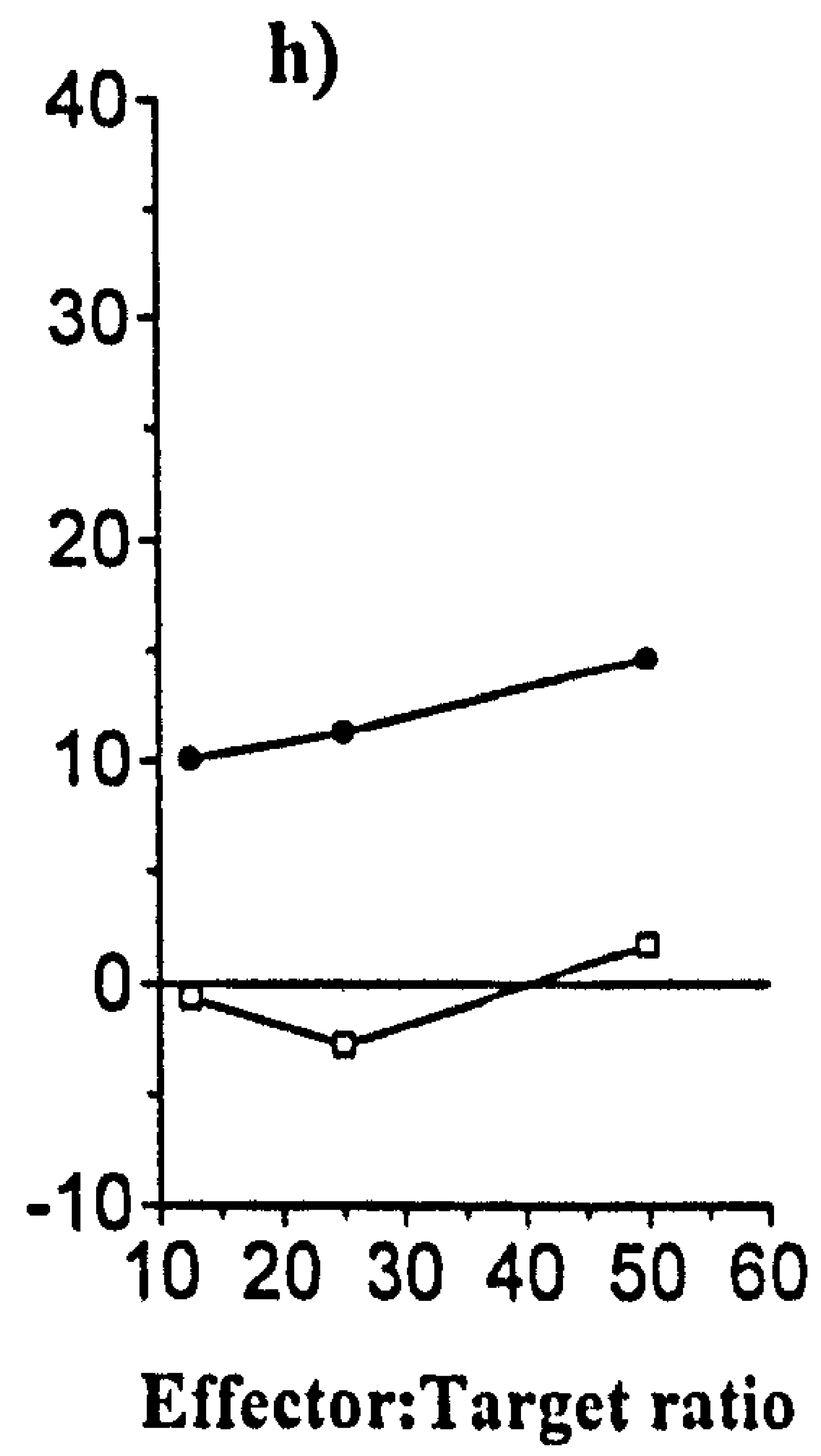
Figure 5.1ii. Bulk culture CTL activity in PBMC from 4 ponies before and 28 days after infection with EHV-4.

e) #3 before infection
f) #3 after infection
g) #4 before infection
h) #4 after infection









Inducing Antigen:

Table 5.7. The precursor frequencies of EHV-4 cross-reactive CTL in PBMC from four ponies before and 10 days after an EHV-4 infection.

Horse number	Reciprocal frequency Before infection	 After infection
#1	125,000	15,625
#2	<150,000	33,333
#3	<150,000	<150,000
#4	<150,000	111,111

After challenge of all four horses with EHV-4, infection could only be demonstrated (by nasal excretion of live virus and/or pyrexic response) in ponies #1 and #2.

These results suggest that ponies #1 and #2 became infected with EHV-4 and cleared the infection by producing an immune response involving both production of antibody and CTL. Pony #3 did not become infected with EHV-4 but did produce a modest antibody response against the virus which may have been stimulated by residual inactivated viral antigen draining into the local lymph nodes from the nasopharynx. Pony #4 did not become infected with EHV-4.

These results show that EHV-4 infection induces CTL *in vivo* but that some animals may be protected from infection, even though the CTLp frequency in PBMC is <150,000.

5.4. Discussion

The humoral immune response to EHV-1 and EHV-4 has been well documented in conventionally reared (Stokes *et. al.*, 1991, Mumford *et. al.*, 1987) and specific pathogen free (SPF) animals (Tewari *et. al.*, 1993, Gibson *et. al.*, 1992a). Conclusions from these studies suggest that the antibody response is predominantly type-specific after EHV-1 infection but that a cross-reactive response develops after subsequent EHV-4 infection. Fitzpatrick and Studdert (1984) studied EHV-1 infection of SPF foals and concluded that, although serological responses were type-specific, cell-mediated responses, as measured by *in vitro* lymphocyte blastogenesis, were cross-reactive. The bulk culture CTL data presented in chapter 3 shows that CTL induced with EHV-4 were able to kill EHV-1 infected target cells, *i.e.* that the CTL were cross-reactive.

Bulk culture CTL assays have their limitations (see chapter 2) in that, after infection, it is often difficult to determine, with certainty, significant differences between % cytotoxicity values derived from different assays. The LDA CTL assay presented in this chapter was developed as a way of quantifying the CTL response of horses to EHV infection. It provides an estimate of the frequency of CTLp whilst retaining the virus-specificity and genetic restriction controls built in to the bulk culture CTL assay. As with the bulk culture assay, EHV-4 was able to induce CTL activity but this virus was not able to infect PWM blast cells to a sufficient level to provide useable target cells. Therefore, CTL primed with EHV-4 *in vivo* and *in vitro* were assayed using EHV-1 infected target cells and were referred to as cross-reactive CTL.

The CTL LDA development experiments showed that, in order to provide optimal conditions for the maturation of CTLp into effector CTL, Mitomycin C treated filler cells and growth factors were necessary additions to the culture. Failure to include these additives in CTL LDA cultures resulted in a reduction in the measured frequency of CTLp and a reduction in the amount of proliferation observed in the culture wells.

The use of this assay to quantify the frequency of CTL against EHV-1 or EHV-4 in a population of PBMC from an immune animal (Table 5.3) showed that the frequencies of CTLp induced with each virus were equivalent. It is likely that the frequency estimates represent the same population of cross-reactive CTL, as only EHV-1 infected target cells were used.

Comparison of CTLp frequencies between viral assay systems is difficult as there are numerous factors which may affect the final estimate. These include the propensity of the virus to cause immune suppression or to replicate in immune cells. However, Schmid (1988) employed LDA to measure the frequencies of CTLp in the blood of humans infected with HSV-1. The CTLp frequencies were reported to be between 1/4000 and 1/8000 although genetic restriction and specificity controls were not employed in this study. Hickling *et. al.* (1987) derived CTLp frequencies from human patients after VZV infection and found that precursors were present at a frequency of about 1/90,000 in an asymptomatic carrier and about 1/15,000 in a subject with recent zoster. The frequencies measured in immune animals using the EHV CTL LDA assay are likely to be lower than if they were measured using the assay developed by Schmid (1988). This is because the virus-specificity and genetic restriction controls employed were not employed by Schmid. However, the frequencies reported are potentially more relevant, as the presence of non genetically-restricted, virus non-specific CTL in PBMC is likely to be a consequence of *in vitro* culture.

The question of whether an EHV-4 infection stimulates cross-reactive immunity and hence protection against infection with EHV-1 has been addressed in a number of studies. Tewari *et. al.* (1993) showed that a true primary infection of SPF foals with EHV-4 strain MD resulted in mild respiratory disease (pyrexia for 1 day and nasal excretion of virus for 5 days). Subsequent infection with EHV-1 strain Ab4, resulted in partial protection as evidenced by nasal excretion of virus on 1 day, pyrexia on 1 day and viraemia in one of 2

animals. The usual course of infection with the same EHV-1 strain in SPF animals was very much more severe than this, with nasal excretion lasting for up to 11 days and a heavy viraemia (Tewari *et. al.*, 1993, Gibson *et. al.*, 1992a). When EHV-1 infected animals were re-infected with the same strain of EHV-1 there was no pyrexia, no viraemia and nasal excretion of virus on only 1 day (2/2 animals). Edington (1990) showed that primary infection of conventionally reared horses with either EHV-1 strain Ab4 or EHV-4 field isolate 2252 did not prevent nasal excretion of live virus after a secondary infection with the homologous virus. Moreover, a subsequent (tertiary) EHV-1 infection of animals given two EHV-4 infections resulted in infection as measured by nasal excretion (mean 5.2 days) and extensive viraemia (mean 2.3 days). In contrast, infection of ponies with EHV-4 after 2 previous EHV-1 infections resulted in no clinical signs *i.e.* full protection against EHV-4 infection.

Taken together, these studies suggest that infection of ponies with EHV-1 is able to induce protection against subsequent infection with EHV-4 whilst infection with EHV-4 only reduces the severity of subsequent EHV-1 infections. The implication is that the immune response stimulated by EHV-4 is either less cross-reactive or of a lower magnitude than that stimulated by EHV-1.

The infection of two multiply infected mares with EHV-1 strain Ab4, presented in this chapter, resulted in only one day of cell-associated viraemia in one of the mares (#219 on day 8 post-infection). Despite very few signs of infection, the CTLp frequency in PBMC

from this mare fell from 1/17,857 to 1/52,632 17 days after infection and then increased to 1/11,764 41 days after infection. The CTLp frequency in PBMC from an uninfected control mare remained undetectable over the first 17 days of the experiment. These results show that adult horses may become infected with EHV-1 but show few or no clinical signs but that these infections are reflected by changes in the frequency of CTLp in the blood. Serological screening would have failed to detect the establishment of subclinical infections in these animals.

Infection of 4 younger animals with EHV-4 strain V1778, again, resulted in very few signs of infection. Two of the 4 animals selected for this study were found to have serological evidence of recent previous exposure (Thomson *et. al.*, 1976a). The highest antibody responses were to EHV-1 rather than EHV-4 which suggested that a recent infection with the former had occurred (Thomson *et. al.*, 1976a). Support for the previous infection of all 4 ponies came from the detection of significant CTL activity (by bulk culture methods) before experimental infection. Hence, ponies #3 and #4 had recently been exposed to EHV-1 (antibody data) whilst all four animals had been exposed to infection with either EHV-1 or EHV-4 (CTL data) some time previously.

After infection, the only signs of disease were pyrexia for one day in ponies #1 and #2 and nasal excretion of virus from pony #1 for one day. This strongly suggests that all ponies had experienced previous infections with EHV, as these signs were reduced when

compared with those seen after a true primary infection with EHV-4 (Tewari *et. al.*, 1993).

The results of the bulk culture CTL assays after induction with EHV-4 showed that CTL activity did not increase for at least 28 days after infection. This may mean that EHV-4 specific CTL were slow to develop (*i.e.* longer than 28 days) or that the bulk culture assay system was not sensitive enough to detect changes in CTL activity after infection.

The frequencies of CTLp induced using EHV-4 MD showed significant rises between 10 and 12 days after infection in ponies #1 and #2. These data show that, the CTL stimulated by infection of ponies with EHV-4 cross-reacted with EHV-1 but that ponies #3 and #4 did not become infected with EHV-4. The frequency of CTLp stimulated by infection of pony #1 with EHV-4 was 1/15,625 which was equivalent to the frequencies stimulated by infection of mares #219 and #302 with EHV-1. However, the frequency of CTLp stimulated by EHV-4 infection of pony #2 was significantly lower than those in mares #219 and #302. This suggests that the CTLp frequency stimulated by infection of a horse depends on a number of factors, *i.e.* the infectious load, previous infection history of the horse and the infecting virus strain.

Although more work needs to be done, the development and use of CTL LDA assays described in this chapter have provided new information on CTL responses in horses after EHV-1 and EHV-4 infection.

6. General Discussion

The work presented in this thesis shows that antigen-specific, genetically restricted, $CD8^+$ cytotoxic T lymphocytes (CTL) can be measured in horses after infection with EHV-1 and that infection with EHV-4 induces cross-reactive CTL which can kill EHV-1 infected target cells.

The bulk culture CTL assay used here was shown to be useful and robust. The induction of CTL in culture was dependent on the amount of live antigen added and was not affected by the addition of recombinant human interleukin 2, which suggests that all of the necessary cytokines were produced in the culture.

Study of CTL activity from a range of horses showed that adult horses, which had not been recently infected, exhibited low levels of activity. After experimental infection, levels of CTL activity rose to a maximum of about 60% specific cytotoxicity in adult horses. In young animals, CTL activity was not demonstrated in conventional horses younger than 10 months, however after experimental infection of two specific pathogen free foals, low levels of anti EHV-1 CTL activity were demonstrated. These results taken together, suggest that horses develop only a weak CTL response after primary infection with EHV-1 which then becomes stronger as more infections or reactivitions occur. In the event of horses receiving multiple experimental infections spaced over a short time period, CTL activity was maintained for over one year.

The cross-reactivity of EHV-4 induced CTL was shown by the lysis of EHV-1

infected target cells. The cross-reactivity or type-specificity of EHV-1 induced CTL remains to be shown and awaits the development of EHV-4 infectable target cells. The cross-reactivity of HSV-2 induced CTL for HSV-1 infected target cells was shown by Carter *et. al.* (1982). In this study, HSV-2 infected target cells were less susceptible to lysis by either HSV-1 or HSV-2 induced CTL. This reduced susceptibility was not due to the lack of expression of viral antigens on the HSV-2 infected target cell. The implications of the induction of cross-reactive CTL for the resolution of disease after EHV-1 or EHV-4 infection are not clear. Further work needs to be done in order to clarify the target antigens involved in the induction of the cross-reactive CTL. A glycoprotein (gG) containing cross-reactive epitopes (defined by antibody binding) has been described (Crabb & Studdert, 1993. Crabb *et. al.*, 1995) but whether this glycoprotein also provides CTL epitopes remains to be determined. Some work reported in chapter 3 of this thesis suggests that the EHV-1 IE protein contains at least one CTL epitope. This has recently been confirmed by Allen (G. Allen, University of Kentucky, personal communication). This is analogous to the murine model of HSV infection in which CTL recognise at least 2 IE protein ICP27 peptides (Banks *et. al.*, 1993).

The experiments presented in chapter 4 of this thesis were designed to answer a number of questions:

1) What is the effect of live vs inactivated EHV-1 on the *in vitro* proliferation of equine PBMC?

2) Is there any difference between the immune reactivity (as defined by

proliferative response) of PBMC from multiply infected and initially infected mares?

3) Does the precursor frequency of antigen-specific PBMC capable of proliferating *in vitro* in response to inactivated EHV-1 correlate with increased resistance to infection?

It is clear that live EHV-1 has an effect on the *in vitro* functions of equine lymphocytes. Proliferation of lymphocytes was depressed to a greater extent when live EHV-1 was included in mitogen and antigen stimulated cultures than when inactivated EHV-1 (iEHV-1) was included. The mechanism for this suppression of lymphocyte function is not clear, however, the proteins of EHV-1 are clearly not directly immunosuppressive as iEHV-1 did not reduce the proliferation of lymphocytes to the same extent as live EHV-1. Griebel *et. al.* (1990) showed that bovine herpesvirus type 1 (BHV-1) was able to induce lytic infections of bovine activated T lymphocytes, which occurred in the absence of any viral replication or detectable expression of viral proteins. Allen *et. al.* (1995) showed that equine activated T lymphocytes were infectable with EHV-1 and that high levels of viral antigen expression occurred. Furthermore, EHV-1 was shown to replicate in these cells as demonstrated by a greater than 10 fold increase in plaque titre of infectious virus 72 hours after infection with 0.1 pfu/cell. These data and the fact that EHV-1 only infects between 10% and 20% of non-stimulated PBMC (see chapter 2) suggests that the cell-associated viraemia seen after EHV-1 infection may come about as a consequence of the virus replicating in *in vivo* stimulated blasting T cells. Further work needs to be done in order to confirm which cells are infected during viraemia.

Data presented here on the *in vitro* proliferation of equine PBMC in response to mitogens after EHV-1 infection of horses shows that the reporting of stimulation indices alone without supporting raw cpm data can be misleading. Also the importance of the collection of full data sets including virological and serological data was shown in chapter 4. The collection of all available data meant that potential relationships could be inferred *e.g.* between peak cell-associated viraemia and suppression of *in vitro* mitogen stimulated proliferative responses in infected mares.

The use of the proliferative LDA assay to measure the precursor frequency of cells capable of proliferating in response to iEHV-1 showed that mares which were protected from abortion after EHV-1 infection had a lower precursor frequency than mares which aborted. Increases in the precursor frequency of these cells occurred after infection in 3 of 4 mares tested. Despite the fact that all relevant controls were not included in this study (*i.e.* there were no parallel cultures containing all constituents except antigen) these data suggest that the measurement of precursor frequencies of proliferative cells alone does not discriminate immune from non-immune animals. This was also the conclusion of Denis *et. al.* (1994) who carried out similar experiments using BHV-1.

In contrast, the CTL LDA method described in chapter 5 distinguished between recently infected and non-infected animals using both EHV-1 and EHV-4 as the stimulating antigen. Conclusions about whether this assay will distinguish between immune and non-immune animals must await its wider use, perhaps using

cryopreserved cells from past challenge infections from which clinical and virological data are available.

In the horses tested to date, frequencies of CTLp in recently EHV-1 infected horses were of the order of between 1/10,000 and 1/20,000 (see Figure 5.4). This was also the case in an immune mare approximately 9 months after infection with EHV-1. A study by Chilmonczyk *et. al.* (1985) showed CTL precursor frequencies of between 1/14,000 and 1/18,000 in children and adults immune to HSV. An interesting finding has been the reduction in CTLp frequency in one mare after infection with EHV-1. This has not been shown using bulk culture CTL assays (Allen *et. al.* 1995).

To my knowledge, this is the first report of the use of LDA techniques in the horse. The application of these methods to the study of CTL precursor frequencies in EHV-1 infections will greatly improve our understanding of the potential of CMI in protection against or recovery from infection. A most valuable measure would be one which correlated with immunity (*i.e.* with protection from clinical disease, virus shedding and cell associated viraemia). If the measurement of anti EHV-1CTLp did correlate with immunity, there could be great potential for the use of CTL LDA as a predictor for the efficacy of both conventional (inactivated whole virus/subunit) or new (deletion mutants, recombinant virus) vaccines.

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Appendix A

This appendix outlines the media formulations used throughout this thesis.

Hanks' Balanced Salts Solution

500ml	1x Hanks' Balanced Salts (modified) without calcium or magnesium. ICN/Flow laboratories (Thame, Oxon) Cat. No. 18-104-54.
1000 units/ml	Benzylpenicillin. Various suppliers.
1000 units/ml	Streptomycin. Evans medical Ltd, Letherhead, Surrey.
0.075%	Sodium Bicarbonate solution. Sigma, Poole, Dorset. Cat. No. S8761.

complete RPMI

500ml	1x RPMI 1640 medium with 2.00g/ml Sodium Bicarbonate, without Glutamine. ICN/Flow laboratories (Thame, Oxon)
Cat.	No. 12-602-54.
2mM	L-Glutamine. ICN/Flow laboratories (Thame, Oxon).
1000 units/ml	Benzylpenicillin. Various suppliers.
1000 units/ml	Streptomycin. Evans medical Ltd, Letherhead, Surrey.
50ml	Foetal Calf Serum. Mycoplasma tested. APP Ltd (Brierley Hill, West Midlands).
5.5×10^{-5}M	2-Mercaptoethanol.

AIM-V/SM Medium

a) 50ml supplemented AIM-V (sAIM-V) medium.

50ml	AIM-V base medium. Life Technologies Ltd, Paisley, Scotland. Cat. No. 12030-029.
2mM	L-Glutamine. ICN/Flow laboratories (Thame, Oxon).
50μg/ml	Gentamycin.
5.5×10^{-5}M	2-Mercaptoethanol.

b) 50ml sensitisation medium (SM) with autologous serum.

44ml	RPMI 1640 base medium.
2mM	L-Glutamine.
50μg/ml	Gentamycin.
5.5×10^{-5}M	2-Mercaptoethanol.
10mM	Sodium Pyruvate.
10mM	MEM non-essential amino acids.
5ml	Heat inactivated, autologous equine serum.

To prepare AIM-V/SM mix 50ml sAIM-v (a) with 50ml SM (b).

Appendix B

Limiting Dilution Analysis

The use of Limiting Dilution Analysis (LDA) techniques in chapters 4 and 5 of this thesis has facilitated the estimation of the frequencies of particular cell types within peripheral blood mononuclear cell (PBMC) populations. This appendix describes the theory behind LDA. Lefkovits and Waldmann (1984) have written an excellent review of the background to LDA.

LDA is a quantitative method which allows the estimation of the frequency of a responding cell type within a mixed population. The technique was first used to estimate the frequencies of antigen-specific B, T helper and T suppressor cells (Lefkovits, 1972. Lefkovits & Waldmann, 1979. Quintans *et. al.*, 1973). Subsequently, LDA has been used to measure the frequencies of antigen-specific and allogeneic cytotoxic T lymphocytes (CTL) and cytokine producing cells (Taracha *et. al.*, 1992. Juretic & Knowles, 1991. Kaminski *et. al.*, 1991. Schulick *et. al.*, 1993., Deacock *et. al.*, 1992).

There is no simple morphological property which may be used to distinguish cells in a population which will respond to antigen from those which will not. It is, however, possible to use the fact that, under favourable conditions, a single immune cell can develop into a measurable clone of cells. In order for this to occur, the responding cell must be cultured with an excess of appropriate accessory cells and growth factors. If this line of reasoning is followed then it is clear that if a population of $1x10^6$ PBMC contains 400 cells capable of responding to an antigen then the frequency of responding cells will be one in 2500. Random distribution of these $1x10^6$ PBMC cells in, say, 400 culture wells

(*i.e.* an average of one cell per well) may result in some wells receiving no cells, some receiving one cell and some receiving two cells *etc*. Each culture well will also receive an excess of other cells from the PBMC population which will have other antigenic specificities and will be functionally silent within this analysis. In this example we would obtain approximately 150 negative cultures (37%) and 250 positive cultures (67%) using a sensitive readout. The reason why a given fraction of negative (and positive) wells is obtained is not straightforward, however, in the common situation where we do not know, but would like to estimate, the precursor frequency of, say, CTL in a PBMC population we assess the number of negative wells at a number of appropriate PBMC inputs and from these values make a frequency estimate. It is not possible to estimate, accurately, the frequency of responding cells from only one dilution. Therefore, in practice, a range of dilutions is used. Provided with a sufficiently sensitive readout system and optimum conditions for growth, it is relatively easy to determine the proportion of responding and non-responding cultures in an experiment.

The proportion of cultures which fail to respond (i.e. failed to receive any CTL) at a given PBMC input is defined by the following equation:

$$F_0 = e^{-u}$$

where F_0 is the fraction of negative cultures, e is the base of the natural logarithm and u is the average number of precursor cells per culture. The above expression is the zero term of the Poisson distribution. The full derivation of the formula and its relationship to the binomial distribution is considered in detail in Lefkovits & Waldmann (1979).

In order to yield an explicit expression of the average number of precursor cells per culture, it is necessary to convert the formula to its logarithmic form:

$$u = -\ln F_0$$

This formula can also be represented graphically, providing a straight line on a semilogarithmic plot. In other words, the negative logarithm of the proportion of negative cultures is linearly proportional to the mean number of precursor cells per culture. The resultant straight line serves two purposes, firstly it allows an estimation of the frequency of responding cells in the original population (see below) and secondly, it serves as an internal control to verify that the system is measuring one limiting cell type (i.e. the curve is a single hit curve). Any deviation from linearity means either that the culture conditions are not optimal for the growth of a single cell type, that the readout failed to detect wells containing single clones or that complex cellular interactions are occurring within the culture well. Under these non-linear situations the data obtained can only be used to inform experimental redesign. It is obvious that visual estimation of the frequency of responding cells and the linearity of the resultant curve is inadequate; therefore statistical methods need to be applied to the experimental data.

Estimation of the frequency of responding cells

As mentioned above, if we plot the negative logarithm of the fraction of non-responding cultures (y axis) against the cell input (x axis), we can expect to obtain a straight line passing through the origin if the criteria outlined above have been met. The size of the original sample containing one precursor cell can be estimated by interpolating at the level of 37% non-responding cultures. This is explained by substituting $u = 1$ into the

zero term of the Poisson distribution, thus:

$$F_0 = e^{-u}$$

Then $$F_0 = e^{-1} = 1/e = 1/2.7$$

$$F_0 = 0.37$$

The choice of the statistical procedure for the calculation of the frequency of responding cells from the experimental data has been discussed widely (Taswell, 1981. Fazekas de St.Groth, 1982). As a result of this discussion three main statistical methods have been advocated. Lefkovits and Waldmann (1979) preferred to use the least squares method which is now thought to be inappropriate for the evaluation of LDA data as this method may reject inherantly valid data (Fazekas de St.Groth, 1982). Taswell (1981) proposed the method of chi-squared minimisation (MC) and likelihood maximisation (ML). Fazekas de St.Groth (1982) also advocated the use of the ML method but raised doubts about the use of the MC method. A comparison of the MC and the ML methods was carried out by Strijbosch *et. al.* (1987) who calculated the mean bias of each method when performing 2000 simulated LDA experiments. The authors concluded that a Jacknife version (see Miller, 1974, for basic definitions of the jacknife) of the ML method originally advocated by Reeds (1978) was the most appropriate for this type of data as it exhibited a mean relative bias of zero for the complete range of frequencies, closely followed by the ML method and, finally the MC method.

Strijbosch *et. al.* (1988) have produced a computer program which provides a frequency estimate based on the ML, Jacknifed ML and MC methods, a 95% confidence interval for each estimation and a goodness-of-fit statistic which should be below published

values using the stated degrees of freedom in order to satisfy the single hit model.

All LDA data presented in this thesis have used this computer program to determine the frequency of responding cells the statistical method of choice being the Jacknife version of the ML method.

Appendix C

This appendix gives examples of the data obtained from two proliferative LDA assays reported in chapter 4.

The design program supplied by Strijbosch *et. al.* (1988) was used to provide initial cell input values, x(i), the number of dilutions, m and the dilution factor, c. These parameters were as follows for the two assays reported:

Horse #302	Before infection	After infection
x(i)	6545	760
m	12	7
c	1.35	1.65

Variations in the design of the proliferative LDA were necessary in order to ensure that the correct range of cell inputs were used. Estimations of the approximate range of frequencies in a population were made using bulk culture proliferation assays as a guide.

The LDA assays were performed as described in chapter 4. The raw data was tabulated using a computer spreadsheet package (Lotus 1-2-3). Tables C.1 and C.2 show the raw data in counts per minute (cpm) from proliferative LDA assays using PBMC from horse #302 before (C.1) and 5 weeks after (C.2) infection with EHV-1 strain Ab4/13. Table C.1 shows that increasing the cell number per well had little effect on the resultant cpm.

Table C.1. An example of the raw data in counts per minute obtained from a proliferative LDA assay.

PBMC from mare #302 before infection.

Responders/well (counts per minute: positives in bold)

29348.00	**21739.00**	**16103.00**	**11928.00**	**8836.00**	**6545.00**	**0**		
44.00	49.00	34.00	36.00	45.00	44.00	58.00		
39.00	35.00	55.00	61.00	41.00	35.00	28.00		
46.00	43.00	31.00	50.00	50.00	46.00	41.00		
64.00	52.00	37.00	47.00	45.00	40.00	43.00		
44.00	50.00	47.00	46.00	38.00	39.00	44.00		
36.00	47.00	38.00	34.00	39.00	31.00	44.00		
36.00	39.00	54.00	40.00	41.00	45.00	51.00		
47.00	47.00	38.00	53.00	35.00	36.00	53.00		
36.00	59.00	37.00	43.00	39.00	38.00	31.00		
43.00	46.00	30.00	41.00	35.00	37.00	43.00		
66.00	38.00	53.00	50.00	45.00	44.00	43.00		
27.00	44.00	48.00	39.00	46.00	48.00	45.00		
40.00	38.00	47.00	38.00	37.00	59.00	50.00		
39.00	43.00	40.00	40.00	35.00	43.00	46.00		
34.00	48.00	43.00	53.00	51.00	55.00	43.00		
46.00	48.00	32.00	50.00	51.00	57.00	32.00		
51.00	32.00	33.00	35.00	55.00	44.00	42.00		
39.00	43.00	36.00	40.00	38.00	33.00	43.00		
29.00	39.00	37.00	42.00	44.00	38.00	35.00		
44.00	45.00	37.00	57.00	32.00	50.00	44.00	**Mean (0)**	**SD x 3**
45.00	30.00	48.00	41.00	40.00	35.00	43.00	43.09	20.64
50.00	54.00	37.00	43.00	51.00	58.00	48.00		
45.00	55.00	42.00	**71.00**	48.00	41.00	41.00	**SD**	**+ve =>**
56.00	36.00	44.00	42.00	46.00	48.00	-	6.88	63.73

Table C.2. An example of the raw data in counts per minute obtained from a proliferative LDA assay.

PBMC from mare #302 5 weeks after infection with EHV-1 Ab4/13.

Responders/well (counts per minute: positives in bold)

9295	5633	3414	2069	1254	760	0		
578.00	**159.00**	**130.00**	**153.00**	39.00	27.00	28.00		
643.00	**307.00**	**127.00**	**78.00**	**100.00**	**65.00**	30.00		
827.00	53.00	**198.00**	**89.00**	**82.00**	33.00	25.00		
480.00	**247.00**	46.00	35.00	32.00	29.00	31.00		
559.00	**136.00**	**67.00**	**75.00**	40.00	28.00	29.00		
134.00	**219.00**	**106.00**	**226.00**	24.00	31.00	36.00		
427.00	**62.00**	**77.00**	56.00	**103.00**	32.00	29.00		
229.00	**350.00**	**74.00**	**102.00**	56.00	32.00	37.00		
522.00	**224.00**	**118.00**	**197.00**	55.00	42.00	32.00		
440.00	**123.00**	**118.00**	**66.00**	34.00	52.00	34.00		
358.00	**401.00**	**93.00**	47.00	**64.00**	40.00	32.00		
354.00	**186.00**	44.00	37.00	39.00	47.00	40.00		
166.00	**104.00**	**111.00**	**62.00**	39.00	46.00	36.00		
398.00	**111.00**	**87.00**	35.00	32.00	49.00	30.00		
218.00	42.00	**76.00**	**146.00**	**99.00**	**62.00**	26.00		
259.00	**175.00**	**88.00**	**87.00**	40.00	37.00	21.00		
642.00	**146.00**	**160.00**	**62.00**	**90.00**	25.00	41.00		
497.00	**467.00**	**140.00**	36.00	**88.00**	41.00	61.00		
241.00	**379.00**	**79.00**	**227.00**	**74.00**	33.00	34.00		
356.00	**146.00**	44.00	40.00	56.00	29.00	43.00	Mean (0)	SD x 3
170.00	**245.00**	47.00	**60.00**	**77.00**	36.00	40.00	34.54	24.63
1651.00	**430.00**	**115.00**	43.00	58.00	41.00	39.00		
234.00	**187.00**	48.00	32.00	**97.00**	47.00	30.00	SD	+ve = >
184.00	**170.00**	**139.00**	33.00	**79.00**	49.00	45.00	8.21	59.17

The number of wells which produced a negative result, *i.e.* less than the mean cpm from the wells containing no responders + 3 standard deviations, were entered into a word processing package (Wordperfect for Windows version 5.2) in the following format:

x(1)_# of replicates per dilution_# of negative wells

x(2)_# of replicates per dilution_# of negative wells

x(3)_# of replicates per dilution_# of negative wells *etc*

This data was saved on a diskette as an ASCII text file. The Evaluate program was opened from the C: prompt. The input file name (*i.e.* the ASCII file name) and the output file names were specified and the evaluation was started. When the evaluation was complete, the output file was opened from the word processor. Examples of the output derived from two LDA evaluations are shown in tables C.3 and C.4. These results tables show the input cell number, x(i), the number of replicates, n(i), the number of negative wells, r(i) and the proportion of negative wells, r(i)/n(i). These data are fitted to a straight line passing through the origin and estimates of the frequency of responding cells are made using the three methods of Maximum likelihood, the jackknifed version of the maximum likelihood and the minimum chi squared (see appendix B). An estimation of the goodness-of-fit of the data to a straight line (*i.e.* single hit kinetics) is produced using the chi squared method. Chi squared values above published values, using the number of dilutions minus 1 as the degrees of freedom, mean that the data must be rejected as not satisfying single hit kinetics. The statistics by which all of these values are derived was reviewed by Lefkovits & Waldmann (1979). An example of data from the proliferative LDA which do not conform to single hit kinetics is shown in table C.5.

Table C.3. An example of the output produced from a proliferative LDA evaluation using the program of Strijbosch.

PBMC from mare #302 before infection.

LDA on No. 302 sampled before EHV-1 infection.

i	x[i]	n[i]	r[i]	r[i]/n[i]
1	6545.00000	24	24	1.0000
2	8836.00000	24	24	1.0000
3	11928.00000	24	23	0.9583
4	16103.00000	24	24	1.0000
5	21739.00000	24	24	1.0000
6	29384.00000	24	22	0.9167
7	39620.00000	24	24	1.0000
8	53487.00000	24	23	0.9583
9	72207.00000	24	22	0.9167
10	97480.00000	24	23	0.9583
11	131597.00000	24	22	0.9167
12	177657.00000	24	21	0.8750

Method	Estimate	St.Error	95% Confidence Interval		Goodn-of-fit	
			Lower	Upper	GoF-X2	DF
Max-Lik	0.000001	0.000000	0.000000	0.000001	5.578	7
Jackkni	0.000001	0.000000	0.000000	0.000001	5.598	7
Min.Chi	0.000001	0.000000	0.000001	0.000002	5.547	8

Table C.4. An example of the output produced from a proliferative LDA evaluation using the program of Strijbosch.

PBMC from mare #302 5 weeks after infection with EHV-1 Ab4/13.

LDA on No. 302 sampled 5 weeks after EHV-1 infection.

i	x[i]	n[i]	r[i]	r[i]/n[i]
1	760.00000	24	22	0.9167
2	1254.00000	24	13	0.5417
3	2069.00000	24	10	0.4167
4	3414.00000	24	5	0.2083
5	5633.00000	24	2	0.0833
6	9295.00000	24	0	0.0000
7	15336.00000	24	0	0.0000

Method	Estimate	St.Error	95% Confidence Interval Lower	Upper	Goodn-of-fit GoF-X2	DF
Max-Lik	0.000423	0.000052	0.000320	0.000526	4.936	4
Jackkni	0.000419	0.000049	0.000323	0.000514	4.947	4
Min.Chi	0.000424	0.000055	0.000315	0.000532	4.936	4

Table C.5. An example of the output from a proliferative LDA when the data did not conform to single hit kinetics.

PBMC from mare #219 21 days after infection with EHV-1 Ab4/13.

LDA on No. 219 sampled 21 days after EHV-1 infection.

i	x[i]	n[i]	r[i]	r[i]/n[i]
1	370.00000	24	24	1.0000
2	562.00000	24	23	0.9583
3	855.00000	24	24	1.0000
4	1299.00000	24	24	1.0000
5	1975.00000	24	24	1.0000
6	3002.00000	24	21	0.8750
7	4563.00000	24	9	0.3750
8	6936.00000	24	5	0.2083
9	10542.00000	24	2	0.0833
10	16025.00000	24	1	0.0417
11	24358.00000	24	0	0.0000
12	37024.00000	24	0	0.0000

Method	Estimate	St.Error	95% Confidence Interval Lower	95% Confidence Interval Upper	Goodn-of-fit GoF-X2	DF
Max-Lik	0.000145	0.000016	0.000114	0.000177	31.857*	9
Jackkni	0.000145	0.000013	0.000120	0.000169	31.882*	9
Min.Chi	0.000152	0.000019	0.000115	0.000188	31.736*	9

Starred Chi-Squared values are significant on the 5% level.

Appendix D

This appendix gives examples of the data obtained from two CTL LDA assays reported in chapter 5.

The design program supplied by Strijbosch *et. al.* (1988) was not used to provide initial cell input values as no prior information was available to inform the design. Instead, the following design was set and used throughout the study:

x(i)	781
m	7
c	2

The LDA assays were performed as described in chapter 5. The raw data was tabulated using a computer spreadsheet package (Lotus 1-2-3). Tables D.1 to D.6 show the raw data in counts per minute (cpm) from CTL LDA assays using PBMC from horse #302 stimulated with EHV-1 strain Ab4/13 (D.1-3) and EHV-4 strain MD (D.4-6). Infected target cells from horse #219 were used as the mis-matched target (see chapter 5).

The number of wells which produced a negative result, *i.e.* when the specific ^{51}Cr release value using the autologous infected target cell was less than 10 above the corresponding well using both the mis-matched and the autologous mock-infected target cell, were entered into a word processing package (Wordperfect for Windows version 5.2) in the following format:

Table D.1. An example of the raw data in % specific cytotoxicity obtained from a CTL LDA assay.

CTL induced using EHV-1 Ab4/13 from mare #302.

Autologous infected target cells.

a)

302 Infected Targets

Responder cells/well (positive wells in bold)

0	50,000	25,000	12,500	6,250	3,125	1,563	781
-2.82	16.27	**21.91**	4.12	**17.57**	1.95	6.29	35.36
-0.65	**32.32**	**34.92**	4.99	4.12	10.20	20.17	0.22
5.42	**39.70**	**47.51**	3.25	13.67	-1.08	5.86	-3.90
1.95	**12.80**	**35.79**	8.03	**17.14**	6.29	10.63	6.29
-0.22	**32.75**	**29.72**	-4.34	7.16	-1.95	-2.39	5.42
2.82	**34.49**	**26.68**	1.95	**19.74**	0.65	0.22	8.46
-1.08	20.17	2.39	11.06	0.22	7.16	5.86	9.33
-1.08	**86.98**	**76.14**	**24.08**	9.76	**92.62**	-3.90	17.14
6.29	35.36	1.08	8.03	-2.39	**8.46**	4.56	-0.22
-2.39	**31.89**	10.20	**21.91**	**12.36**	5.86	4.99	-8.46
13.23	**25.81**	**73.54**	10.20	-0.22	0.65	16.27	8.46
-2.39	**31.45**	**20.17**	4.99	1.95	0.65	9.33	-0.22
-5.42	**22.78**	24.95	1.52	6.29	**11.93**	9.76	0.22
-3.90	**11.93**	14.53	**20.61**	-6.72	8.46	10.63	-3.90
-2.39	**31.89**	8.46	1.08	8.03	8.89	7.16	6.72
-11.50	**36.23**	14.53	10.20	11.50	**15.84**	-4.56	-1.95
-5.86	**28.42**	14.53	0.22	**17.57**	-1.52	8.89	1.95
4.12	18.87	15.40	6.29	-1.52	-0.65	10.20	-2.82
-7.16	**52.71**	**14.53**	**38.39**	**21.91**	1.52	13.23	4.99
-1.52	**19.31**	**26.68**	11.06	10.63	2.82	-3.90	0.65
-0.65	**19.74**	**26.68**	5.86	7.16	1.95	3.69	-8.89
-12.36	**15.40**	**28.85**	8.03	-5.42	**16.70**	-3.90	-8.03
-1.52	**21.91**	**26.68**	**22.78**	13.67	-4.34	-1.95	-0.22
-8.46	**14.10**	**17.14**	**66.16**	-1.52	3.25	9.76	7.16

	0	50,000	25,000	12,500	6,250	3,125	1,563	781
Mean	-1.56	28.89	25.54	12.10	7.61	8.18	5.70	3.07
St. Dev.	5.65	15.87	18.57	14.98	8.26	18.83	6.75	9.32

Table D.2. An example of the raw data in % specific cytotoxicity obtained from a CTL LDA assay.

CTL induced using EHV-1 Ab4/13 from mare #302.

Allogeneic (MHC class I mis-matched) infected target cells.

b)

219 (mis-matched) Infected Targets

Responder cells/well

	0	50,000	25,000	12,500	6,250	3,125	1,563
	0.22	-3.56	-5.33	4.89	-4.89	8.44	-0.89
	1.78	12.89	1.33	8.00	3.11	0.67	6.22
	5.33	4.44	2.67	4.44	5.33	4.89	-2.22
	4.89	0.67	3.11	-8.44	3.56	10.67	3.11
	3.11	1.78	15.56	4.00	10.67	-0.44	-7.56
	-0.44	4.89	7.56	-1.78	-4.00	-0.44	7.56
	-1.78	-1.33	4.89	-7.56	4.44	7.11	4.44
	4.00	7.11	28.00	8.89	-1.78	11.11	-0.44
	-5.33	27.11	-2.67	5.78	-0.89	-6.22	-1.33
	-1.33	2.67	8.44	4.00	-6.22	-3.56	-2.22
	4.89	5.33	-4.89	-3.11	0.00	0.67	6.22
	10.67	6.67	-0.44	-5.78	-8.00	4.00	5.78
	4.44	6.22	5.33	-0.89	-1.78	1.33	-
	12.89	8.44	-3.11	0.67	0.67	6.22	-
	-2.22	16.89	8.89	-8.00	5.33	15.11	-
	0.00	4.89	4.89	-9.33	8.89	-9.78	-
	5.33	2.67	1.78	0.00	0.22	-0.44	-
	-4.00	8.89	8.00	-2.22	5.33	6.67	-
	-6.67	-4.89	-1.78	25.33	-1.78	12.44	-
	4.44	-8.00	13.78	-2.22	-1.33	5.33	-
	4.89	9.33	12.44	9.33	2.22	2.22	-
	-7.11	0.22	13.33	0.00	-1.78	-5.78	-
	0.00	5.78	4.89	-0.44	4.89	7.11	-
	0.67	0.00	4.44	13.33	-8.89	7.11	-
Mean	1.61	4.96	5.46	1.62	0.56	3.52	1.56
St. Dev.	4.95	7.27	7.57	7.89	5.02	6.21	4.65

Table D.3. An example of the raw data in % specific cytotoxicity obtained from a CTL LDA assay.

CTL induced using EHV-1 Ab4/13 from mare #302.

Autologous mock-infected target cells.

c)

302 Mock Infected Targets

Responder cells/well

	0	50,000	25,000	12,500	6,250	3,125	1,563
	1.73	15.58	5.58	-8.27	5.19	-	-
	1.73	7.12	6.73	1.35	4.42	-	-
	2.12	-0.19	10.58	0.96	-0.19	-	-
	4.42	0.96	6.35	7.50	1.35	-	-
	-0.96	4.04	7.12	3.65	8.27	-	-
	7.88	7.12	7.12	10.96	5.96	-	-
	5.58	11.73	5.58	7.12	14.04	-	-
	-0.19	4.04	24.04	12.12	2.50	-	-
	3.27	7.88	0.19	8.65	9.04	-	-
	-1.35	-0.19	3.27	7.50	0.96	-	-
	0.19	5.58	6.35	3.27	-1.35	-	-
	-0.19	8.65	7.12	2.88	5.19	-	-
	2.50	10.19	18.65	-2.88	11.73	-	-
	-1.73	-0.58	5.58	-0.58	2.88	-	-
	3.27	18.65	15.58	-1.73	11.35	-	-
	4.04	-0.58	12.12	1.35	12.12	-	-
	-0.96	-2.88	9.04	-0.96	4.04	-	-
	3.27	-1.73	5.58	11.35	14.42	-	-
	2.12	14.81	-4.04	15.19	6.73	-	-
	-1.35	3.65	-2.88	12.12	3.65	-	-
	1.73	9.42	2.12	4.42	-5.58	-	-
	10.19	0.58	7.12	-4.04	-3.65	-	-
	9.81	8.65	10.19	0.96	3.65	-	-
	9.42	-4.04	3.65	49.04	5.96	-	-
Mean	2.77	5.35	7.20	5.91	5.11	0.00	0.00
St. Dev.	3.60	6.16	6.17	10.90	5.27	0.00	0.00

Table D.4. An example of the raw data in % specific cytotoxicity obtained from a CTL LDA assay.

CTL induced using EHV-4 MD from mare #302.

Autologous infected target cells.

a)

302 Infected Targets

Responder cells/well (positive wells in bold)

	0	50,000	25,000	12,500	6,250	3,125	1,563	781
	-8 46	47.07	26.68	34.49	37.53	-2.82	11.93	-3.90
	-7.16	45.34	31.45	34.92	18.87	1.08	-1.08	4.12
	-1.52	4.56	1.52	37.09	5.86	1.52	-1.08	-1.52
	3.25	46.64	21.48	2.39	4.12	30.59	0.65	1.08
	2.39	5.86	34.92	34.49	0.65	2.39	-3.25	-2.39
	-10.20	48.37	49.24	34.06	16.27	0.65	0.22	5.86
	-3.90	37.09	16.27	7.16	0.22	-7.16	-3.90	-2.39
	-7 59	22.78	6.29	12.80	0.65	11.50	10 20	2.82
	-3.25	37.53	8.89	15.40	-0.65	2.39	-4.99	-4.34
	-5.86	5.42	18.87	-10.63	-9.76	7.16	-6.72	4.12
	-5.42	37.53	8.03	38.83	10.20	8.03	4.56	-5.86
	-4.34	111.71	17.57	30.15	-0.65	4.56	-7.59	-8.03
	-1.95	17.57	21.48	10.20	0.22	1.52	0.65	-0.65
	8.03	27.55	19.31	47.51	8.03	1.95	0.00	-1.52
	-0.65	1.95	43.17	8.46	0.22	0.22	-7.16	-2.82
	8.89	57.92	50.54	10.20	-4.99	17.57	4.56	4.99
	0.22	17.14	4.12	36.23	-5.86	0.22	1.95	-6.29
	1.52	40.56	47.07	6.29	-0 65	-3.25	2.39	-2.39
	0.65	39.70	24.95	-1.52	-5 42	-2.39	-0.65	0.22
	0.65	40.13	14.97	12.80	14.53	-2.39	-1.95	-6.72
	4.56	50.54	31.45	14.53	-1.52	3.69	-3.90	3.69
	6.72	43.60	24.95	7.59	0.22	0.65	-3.90	-1.52
	7.16	43.60	9.33	10.63	23.64	-4.56	33.62	1.08
	-6.72	94.79	49.67	-1.08	-4.99	0.65	1.08	6.72
Mean	-0.96	38 54	24.26	18.04	4.45	3.07	1.07	-0.65
St. Dev.	5.53	25.80	15 17	15.81	10 95	7.87	8 46	4.18

Table D.5. An example of the raw data in % specific cytotoxicity obtained from a CTL LDA assay.

CTL induced using EHV-4 MD from mare #302.

Allogeneic (MHC class I mis-matched) infected target cells.

b)

219 (mis-matched) Infected Targets

Responder cells/well

	0	50,000	25,000	12,500	6,250	3,125	1,563
	1.78	-2.22	11.11	0.00	1.33	5.33	-1.78
	-11.56	4.00	-1.78	-4.89	8.00	-0.89	8.44
	1.33	-8.44	0.00	-12.44	-10.67	-0.89	-7.56
	-4.44	10.67	-3.11	2.67	-4.44	3.56	2.22
	-12.44	2.22	-4.89	-7.11	-4.89	8.44	-7.11
	-7.56	-3.11	-8.00	-6.67	-1.78	-1.78	-2.67
	-10.67	0.67	0.00	1.78	-10.67	4.89	-5.33
	8.00	-1.33	-1.78	0.22	1.33	3.11	2.67
	-3.11	-10.67	3.11	-7.56	-2.22	2.22	-3.11
	-2.67	-7.11	4.44	-8.00	1.78	0.22	-11.11
	-7.56	0.00	-8.44	8.00	-7.56	0.67	6.22
	-10.67	-3.56	-4.89	1.78	0.67	-4.44	0.00
	-0.89	11.56	-5.33	-4.44	-5.33	-2.67	-
	-3.56	-4.00	0.22	0.00	3.56	2.22	-
	-3.56	0.00	17.33	-5.33	-8.89	-0.89	-
	0.22	0.00	16.00	7.11	-1.33	-0.44	-
	-8.89	7.56	2.22	-4.44	-1.33	-8.00	-
	-3.56	0.00	8.44	-3.11	2.67	-0.89	-
	2.67	3.11	-11.11	-6.67	0.00	3.56	-
	-4.00	0.67	-1.78	-12.44	-5.78	-1.33	-
	-1.33	4.44	-12.44	-8.44	7.11	4.89	-
	-3.11	-6.22	-1.78	-4.89	-3.11	-7.11	-
	4.00	1.33	-7.11	3.56	-8.89	-0.89	-
	5.78	6.22	3.56	2.67	-5.78	0.00	-
Mean	-3.16	0.24	-0.25	-2.86	-2.34	0.37	-1.59
St. Dev.	5.51	5.57	7.69	5.59	5.16	3.85	5.81

Table D.6. An example of the raw data in % specific cytotoxicity obtained from a CTL LDA assay.

CTL induced using EHV-4 MD from mare #302.

Autologous mock-infected target cells.

c)

302 Mock Infected Targets

Responder cells/well

	0	50,000	25,000	12,500	6,250	3,125	1,563
	7.50	12.12	4.81	0.96	3.65	-	-
	-2.50	6.73	-0.58	6.73	4.04	-	-
	4.42	-8.65	1.73	-1.73	6.73	-	-
	3.27	16.73	-2.12	5.96	3.65	-	-
	-4.04	2.88	3.65	0.19	10.19	-	-
	4.81	6.73	0.96	-0.58	1.73	-	-
	1.73	2.12	-3.65	-6.92	-1.73	-	-
	-2.12	-0.19	3.27	6.73	7.50	-	-
	3.27	6.73	-4.42	-	2.50	-	-
	0.96	-8.27	0.19	-4.04	-3.65	-	-
	-4.81	3.65	2.50	5.19	5.58	-	-
	6.35	0.19	-1.73	2.50	5.96	-	-
	-4.42	2.12	8.27	2.12	-3.27	-	-
	9.81	-4.04	13.27	-1.73	9.81	-	-
	9.04	9.04	4.04	4.81	1.35	-	-
	5.58	13.65	16.35	4.42	0.58	-	-
	5.19	-1.35	5.58	-1.73	7.88	-	-
	1.35	0.19	-0.58	6.73	-0.19	-	-
	-4.42	9.42	-4.81	-2.50	3.65	-	-
	3.27	2.88	-24.81	4.04	-4.81	-	-
	-2.50	3.27	3.65	-6.35	5.58	-	-
	0.58	-0.96	-24.81	4.81	-1.35	-	-
	-0.58	2.12	5.58	-2.12	-1.73	-	-
	0.96	3.65	11.35	-2.12	7.88	-	-
Mean	1.78	3.37	0.74	1.06	2.98	0.00	0.00
St. Dev.	4.34	6.18	9.49	4.14	4.34	0.00	0.00

x(1)_# of replicates per dilution_# of negative wells

x(2)_# of replicates per dilution_# of negative wells

x(3)_# of replicates per dilution_# of negative wells *etc*

This data was saved on a diskette as an ASCII text file. The Evaluate program was opened from the C: prompt. The input file name (*i.e.* the ASCII file name) and the output file names were specified and the evaluation was started. When the evaluation was complete, the output file was opened from the word processor. Examples of the output derived from the two LDA evaluations are shown in tables D.7 and D.8. These results tables show the input cell number, x(i), the number of replicates, n(i), the number of negative wells, r(i) and the proportion of negative wells, r(i)/n(i). These data are treated in the same way as the proliferative LDA data in appendix C.

Table D.7. An example of the output produced from a CTL LDA evaluation using the program of Strijbosch.

CTL induced using EHV-1 Ab4/13 from PBMC from mare #302.

#302 CTL LDA induced with EHV-1 Ab4.

#219 mis-matched, infected and autologous mock infected control target cells.

i	x[i]	n[i]	r[i]	r[i]/n[i]
1	3125.00000	24	19	0.7917
2	6250.00000	24	18	0.7500
3	12500.00000	24	18	0.7500
4	25000.00000	24	9	0.3750
5	50000.00000	24	5	0.2083

Method	Estimate	St.Error	95% Confidence Interval Lower	Upper	Goodn-of-fit GoF-X2	DF
Max-Lik	0.000036	0.000005	0.000025	0.000046	4.729	4
Jackkni	0.000035	0.000006	0.000024	0.000046	4.769	4
Min.Chi	0.000036	0.000005	0.000027	0.000046	4.699	4

Table D.8. An example of the output produced from a CTL LDA evaluation using the program of Strijbosch.

CTL induced using EHV-4 MD from PBMC from mare #302.

#302 CTL LDA induced with EHV-4 MD.

#219 mis-match, infected and autologous mock infected control target cells.

i	x[i]	n[i]	r[i]	r[i]/n[i]
1	3125.00000	24	22	0.9167
2	6250.00000	24	19	0.7917
3	12500.00000	24	13	0.5417
4	25000.00000	24	7	0.2917
5	50000.00000	24	4	0.1667

Method	Estimate	St.Error	95% Confidence Interval		Goodn-of-fit	
			Lower	Upper	GoF-X2	DF
Max-Lik	0.000042	0.000006	0.000030	0.000054	1.454	4
Jackkni	0.000041	0.000006	0.000029	0.000053	1.456	4
Min.Chi	0.000041	0.000006	0.000030	0.000053	1.452	4

Abbreviations

ADCC	antibody directed cell mediated cytotoxicity
APC	Antigen presenting cell
BHV	Bovine herpes virus
BSA	Bovine serum albumin
β_2M	β_2 microglobulin
C9	the 9th component of the complement cascade
CD	Cluster of differentiation
CMI	Cell mediated immunity
Con A	Concanavalin A
cpm	counts per minute
cRPMI	RPMI base medium + 10% foetal bovine serum
CTL	Cytotoxic T lymphocytes
CTLp	Cytotoxic T lymphocyte precursors
CyA	Cyclosporin A
DNA	Deoxyribonucleic acid
DTH	delayed type hypersensitivity
EBV	Epstein Barr virus
EHV	Equine herpes virus
EIAV	Equine infectious anaemia virus
ER	endoplasmic reticulum
FACS	Fluorescence activated cell sorter
FBS	Foetal bovine serum
G + C	Guanine and Cytosine
GM-CSF	Granulocyte and Macrophage colony stimulating factor
HBSS	Hanks' Balanced Salts Solution
hCMV	Human Cytomegalovirus
HIV	Human immunodeficiency virus
HSV	Herpes Simplex virus
ICAM	inter cellular adhesion molecule
IEF	Isoelectric focusing
IFN	interferon

Ig	Immunoglobulin
Ii	invariant chain associated with the MHC class II
IL	interleukin
K cells	Killer cells
K562	NK sensitive cell line
KLH	Keyhole Limpet haemacyanin
LAK	Lymphokine activated killer cell
LCMV	Lymphocytic choriomeningitis virus
LDA	Limiting dilution analysis
LFA	lymphocyte function associated antigen
MC	Minimum Chi squared
mCMV	Murine Cytomegalovirus
MHC	Major histocompatibility complex
ML	Maximum Likelihood
MLR	Mixed lymphocyte reaction
moi	multiplicity of infection
NK cell	Natural Killer cell
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PM	peritoneal macrophages
PMA	Phorbol Myristate Acetate
PMN	Polymorhonuclear neutrophil
Prot A	Staphylococcal Protein A
PRV	Pseudorabies virus
PWM	Pokeweed mitogen
RNA	Ribonucleic acid
SFV	Smeliki Forest virus
SI	Stimulation index
SPF	specific pathogen free
TBS	Tris buffered saline
TCGF	T cell growth factor
$TCID_{50}$	50% of the tissue culture infective dose

TcR	T cell receptor complex
TGF	Transforming growth factor
Th	T helper cell
tk	Thymidine Kinase
TNF	tumour necrosis factor
VEC	Vascular endothelial cell
VLA	very late antigen
VSV	Vesicular stomatitis virus
VZV	Varicella Zoster virus

Addendum

The results presented in Table 4.5 were based on raw data derived from scintillation counting. The raw counts per minute (cpm) used to derive these data were generally less than 1000, each sample having been counted for 1 minute only. The low level of the raw cpm means that the data may have been influenced by random events. Therefore, the results in Table 4.5 should not be taken to be significant. A sample of the raw data to which these concerns apply, is given in appendix C (Tables C.1 and C.2).