The Woodchuck as an Animal Model for the Study of the Immune Response in Hepadna Viral Infection

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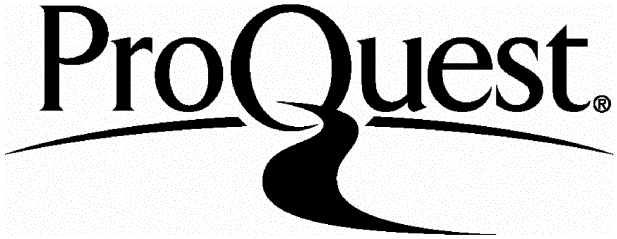
A thesis submitted for the degree of
Doctor of Philosophy in accordance with the regulations of
the University of London

February 1998



DEDICATION

To Mum, for your love,

for your sacrifices, for your indomitable will,

Thank you.

Acknowledgements

I would like to thank Dr. J.A. Waters and Professor H.C. Thomas for their support and assistance during the course of this study and Dr. M. Thursz and Dr. P. Karayiannis for their help with the animals. I gratefully appreciate the help and advice given to me by all members of the department, both past and present, on many aspects of this work. I remain grateful to the British Liver Trust for their financial support and to Dr. Scott Crowe and colleagues at GlaxoWellcome for their contributions. Finally, I would like to thank my family, without whose constant support and encouragement I could not have completed this work.

Abstract

The American woodchuck (Marmota monax) is naturally infected in the wild with a virus called WHV which is similar to HBV. WHV infection causes acute and chronic hepatitis eventually leading to HCC in the majority of cases; the hepatitis is thought to be mediated by the same immune mechanisms involved in HBV infection of humans although this is not yet proven. These similarities make the woodchuck an appropriate model for the study of HBV immunopathogenesis. Lack of information on the woodchuck immune response led to the development of T cell proliferative assays which were initially attempted using conditions suitable for culturing human PBLs. The conditions and kinetics of the T cell response to mitogens was found to differ from humans and other sciurid rodents. This technique was used to investigate the T cell responses to WHV nucleocapsid peptides; an immunodominant epitope was identified in 5 out of 12 chronically infected animals.

In addition to devising an assay to assess T cell responses the crossreactivity that exists between WHeAg and HBeAg enabled employment of a commercial assay to quantify WHeAg levels. Four parameters could now be used to monitor the effects of treatments- WHV DNA, WHeAg, T cell responses and GGT levels. The treatments attempted in this study targeted mainly the cellular arm of the immune response ranging from the use of chemical immunomodulators, such as tucaresol and MPC-866, to the infusion of T cells. T cell adoptive transfer and IL-12 were new treatments tried for the first time in this model. IL-12 was found to increase the proliferative response of woodchuck PBLs with and without PHA in vitro. The woodchuck can be

used to further investigate the usefulness of these and other putative therapies of HBV infection.

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ABBREVIATIONS

aa	amino acid
Ab	antibody
ALT	alanine transaminase
anti-HBc	hepatitis B virus core antibody
anti-HBe	hepatitis B virus e antibody
anti-HBs	hepatitis B virus surface antibody
anti-WHc	woodchuck hepatitis virus core antibody
anti-WHe	woodchuck hepatitis virus e antibody
anti-WHs	woodchuck hepatitis virus surface antibody
AST	aspartate transaminase
ATP	adenosine triphosphate
bp	base pairs
BrdU	5'-bromo-2-deoxyuridine
BSA	bovine serum albumin
C-terminus	carboxy terminus
cDNA	complementary DNA
Ci	Curie
cpm	counts per minute
CTL	cytotoxic T lymphocyte
dATP	adenosine deoxyribonucleside triphosphate
dCTP	cytidine deoxyribonucleoside triphosphate
dGTP	guanosine deoxyribonucleoside triphosphate
dTTP	thymine deoxyribonucleoside triphosphate

DDT	dithiothreitol
DMSO	dimethyl sulphoxide
dNTPs	equimolar mixture of dATP, dCTP, dGTP, dTTP
EDTA	ethylenediaminotetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
g	gram
GGT	gamma glutamyltranspeptidase
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HDV	hepatitis D virus
IgG	immunoglobulin G
IgM	immunoglobulin M
Il	interleukin
kb	kilobase pairs
kD	kilodaltons
2-ME	2-mercaptoethanol
mg	milligram
ml	millilitre
mM	millimolar
mRNA	messenger RNA

MS	mouse serum
N-terminus	amino terminus
ng	nanogram
nM	nanomolar
nm	nanometer
nmol	nanomole
ORF	open reading frame
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
pmol	picomole
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
s.d.	standard deviation
SDS	sodium dodecyl sulphate
S.E.M.	standard error of the mean
S.I.	stimulation index
TE	Tris-EDTA buffer
Tdr	tritiated thymidine
TH	T helper cell
tRNA	transfer RNA
Udr	tritiated uridine

ug	microgram
ul	microlitre
UV	ultraviolet
W	woodchuck (number)
WCS	woodchuck serum
WHcAg	woodchuck hepatitis virus core antigen
WHeAg	woodchuck hepatitis virus e antigen
WHsAg	woodchuck hepatitis virus surface antigen
WHV	woodchuck hepatitis virus

Chapter 1
Introduction

Woodchuck hepatitis virus (WHV) is a member of the family Hepadnaviridae to which HBV also belongs as do other closely related animal viruses e.g. duck hepatitis B virus (DHBV) (Marion et al 1983), ground squirrel hepatitis virus (GSHV) (Marion et al 1980), heron viruses (Sprengel et al 1988) and tree squirrel viruses (Feitelson et al 1986). The family can be subdivided into two genera : *Orthohepadnaviruses*, comprising the mammalian viruses, and *Avihepadnaviruses*, to which the avian viruses belong i.e. DHBV and heron viruses. The former differ from the latter in their nucleotide and amino acid sequence in that *orthohepadnaviruses* possess an additional X gene of unknown function and code for three surface proteins as opposed to two in *avihepadnaviruses*. The family share several common features, one of which is a narrow host range within which a strong hepatotropism is exhibited giving rise to infectious virus and non-infectious viral particles which can be detected in high concentrations in the serum. Acute and chronic hepatitis are characterized by the presence of hepatocyte necrosis, inflammation, lymphocyte infiltration, complications arising from immune complex deposition and eventually hepatocellular carcinoma (HCC).

The hepadnaviruses also demonstrate morphological similarities, particularly virion size and ultrastructure. Both HBV and WHV consist of a nucleocapsid made up of repeating subunits surrounded by a lipid membrane containing the viral envelope proteins. There is considerable nucleotide sequence homology between the two viruses and some antigenic cross-reactivity exists. In addition, virion genome size, structure and genetic organization are comparable. This family of DNA viruses are also unique in that their mode of replication requires reverse transcription of an RNA pregenome and a DNA polymerase is present within the virion.

1.1 Epidemiology

The hepatitis B virus (HBV) is the ninth leading cause of death, globally killing nearly 5% of the Earth's population each year, through the development of chronic hepatitis, cirrhosis and primary hepatocellular carcinoma. An estimated 350 million people are chronically infected carriers of HBV which is a primary cause of death in many countries in Asia, sub-Saharan Africa and the Pacific Basin. In these areas of high endemicity, where the carrier prevalence is 8-15%, there is more than one mode of transmission e.g. in Africa child to child transmission is common and in China mother to child transmission occurs; perinatal transmission also occurs in areas of intermediate endemicity such as Eastern Europe, Japan, the former USSR, Northern South America, where the carrier prevalence is 2-7%. In North America, Australasia, Southern South America and Western Europe, which are classified as areas of low endemicity because the prevalence of HBV infection is less than 2%, high risk groups have been identified. They are :- intravenous drug users, homosexuals, heterosexuals with multiple partners and dialysis patients

The adult woodchuck is covered in coarse grey or reddish brown fur and can weigh between 2 and 6 kilograms with a length ranging between 45 to 65 centimetres. During the hibernation months, between mid-October to mid-February, woodchucks live in their underground dens and emerge before the mating season which occurs during February and March. After a gestation period of 32 days female woodchucks (dams) give birth to single litters of two to seven young woodchucks (pups) who they suckle for approximately six weeks.

The eastern woodchuck, *Marmota monax*, is a hibernating rodent and a member of a family of mammals termed Sciurdae to which squirrels, marmots and chipmunks also belong. The woodchuck is known by two other common names - the groundhog and whistle pig, and is found in the eastern part of the United States and distributed throughout Canada and Southern Alaska. There are other species of woodchuck which are native to North America, the Alps and north of the Himalayas in central Asia. The woodchuck is naturally infected in the wild with a virus similar to HBV, termed woodchuck hepatitis virus (WHV) (Summers et al 1978). Persistent infection with WHV results in chronic hepatitis and ultimately HCC. A high carrier prevalence of 40% exists in woodchucks from south east Pennsylvania, New Jersey, Delaware and Maryland where the infection is hyperendemic (Wong et al 1982, Millman et al 1984) whereas in New York the infection rate is lower (Tennant et al 1988).

1.2 Causative Agents

1.2.1 HBV

HBV was first identified by Blumberg in 1963 when the blood of an Australian Aborigine was found to contain an antigen (Australian antigen) which reacted with sera from American haemophiliacs. In 1970 D.S. Dane revealed its viral origins by immuno-electron microscopy showing the presence of three types of particle : the 42nm completely enveloped virion (termed Dane particles), 20nm spheres present in 10^3-10^6 fold excess over the complete virion and thirdly filaments of variable length with a diameter of 20nm. The HBV virion itself is composed of an outer lipoprotein membrane termed the envelope which surrounds an inner capsid or core, inside which is located the DNA genome as illustrated in figure 1. The three glycoprotein antigens, termed the small, middle and large hepatitis B surface antigen (HBsAg), are displayed

on the surface of all three particles. The spheres and filaments are non-infectious as they are composed of HBsAg and host derived lipids but lack the viral DNA. They are present in the serum in large quantities, approximately 50-300ug HBsAg per ml of serum.

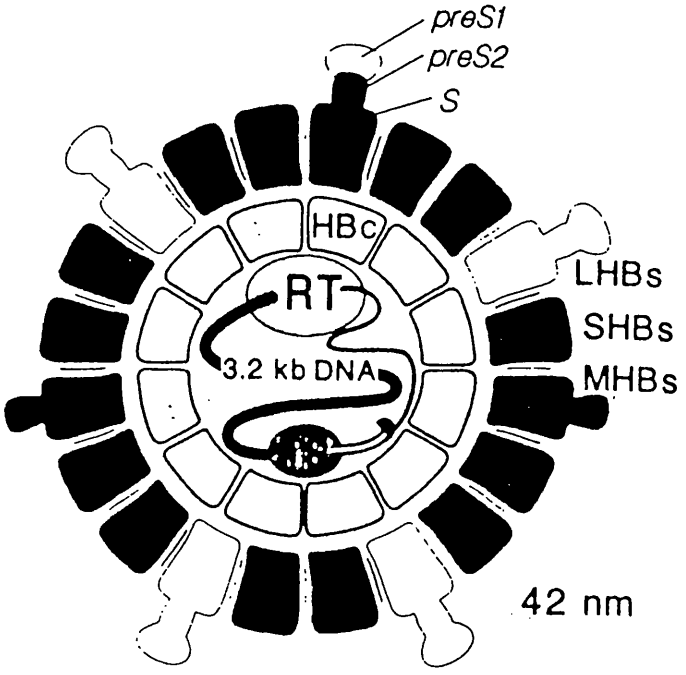


Figure 1. Stuctural Organization of the HBV Genome. From Hess (1993)

The HBV virus contains a partially double-stranded circular piece of DNA which is 3.182 kb in length. Its size makes it one of the smallest animal DNA viruses discovered thus far. The full length strand of DNA is designated the minus strand and is complementary to the viral mRNAs. The incomplete plus strand has a fixed 5' end but the 3' end is variable. The gap region therefore varies between 20-80% of unit length. A cohesive 5' terminus of 224 base pairs ensures the circularity of the duplex. In addition to this asymmmetry the minus strand possesses a covalently linked protein

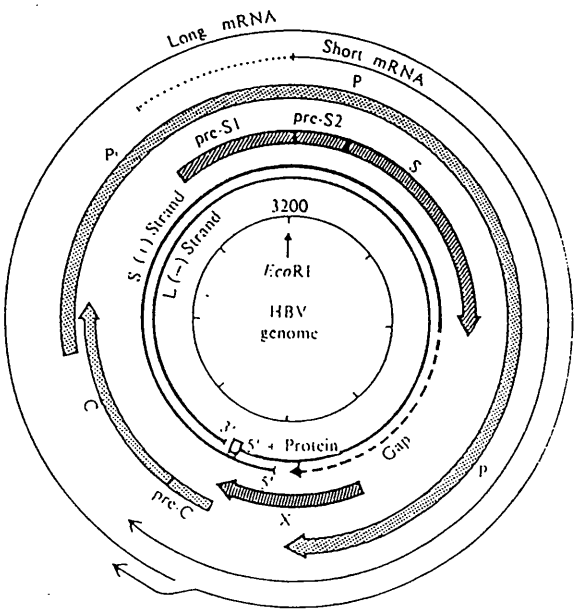
at its 5' terminus whereas the plus strand possesses a short RNA oligomer. The minus strand DNA encodes four major open reading frames (ORFs), pre-core/core, pre-S1/pre-S2/S, P and X. There is a large degree of overlap in the genome because of its size, which ensures that it is used efficiently. Each nucleotide codes for at least one protein and 50% of the genome can be read in more than one frame, often with more than one ATG which produces multiple protein products. ORF-S, the coding region for HBsAg, is located downstream of ORF-pre-S which possesses two in frame ATG codons. This allows transcription of two functional subregions termed pre-S1 and pre-S2 which form surface protein products located on the virion envelope. ORF-C codes for HBcAg, and by proteolytic digestion of pre-C/C, HBeAg. ORF-P is believed to encode the viral polymerase which is involved in the replicative cycle and is covalently attached to the minus strand of DNA via its terminal protein. The ORF-X gene product is thought to regulate viral gene transcription levels (Twu and Schloemer 1987, Arii et al 1992). The two direct repeats, DR1 and DR2 found at the terminal end of the plus strand, are short sequences 10-12 nucleotides in length which are involved in the initiation of viral replication.

1.2.2 WHV

The genomes of several WHV isolates have been cloned and sequenced. The work undertaken in this study utilized genome WHV 8 which was cloned and sequenced by Girones et al (1989) who found the size of 3323 nucleotides identical to the previously published genome sizes of WHV 1 (Galibert et al 1982), WHV 7 and WHV 59 (Cohen et al 1988). This is slightly larger than the HBV genome, as seen in figure 2, with which WHV shares approximately 66% nucleotide sequence homology. The genome has a full length strand of DNA which is complementary to the viral mRNAs and a shorter complementary DNA (cDNA) strand which varies in length.

The four known genes are located on this DNA: core, surface, polymerase and X in addition to two other ORFs, namely ORF-5, overlapping the amino terminus of the X gene and carboxy terminus of the polymerase gene, and ORF-6 which also overlaps the polymerase and X genes but is situated on the complementary DNA strand. Girones et al (1989) found a difference in nucleotide sequence between the isolates: 0.5% between WHV 8 and WHV 7, 1.5% between WHV 8 and WHV 59 and 3.1% between WHV 8 and WHV 1 genomes. In related genomes WHV 2 and WHV 8 i.e. derived from a single parental genome, differences in nucleotide sequence (0.4%) were thought to represent natural variation. Among HBV isolates of the same subtype the variation is similar to woodchucks (1.5-2.0%) but much less than between different subtypes (Okamoto et al 1987,1987).

A.



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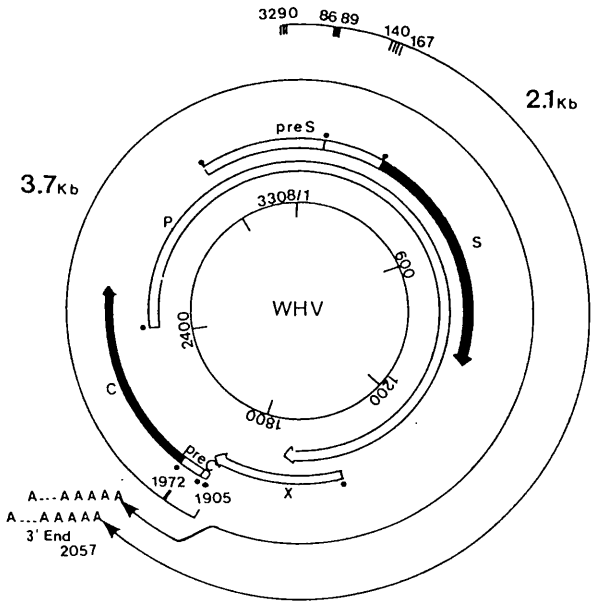

Figure 2. Comparison of the Genomic Organization of A) HBV and B) WHV.

1.3 Mechanism of Replication

Much of what is known about replication was first discovered from work on DHBV which replicates via an RNA intermediate (Summers and Mason 1982). HBV was subsequently shown to have a similar pattern of replicative intermediates (Foster et al 1991) suggesting a similar replication strategy with retroviruses e.g. HIV (Miller and Robinson 1986).

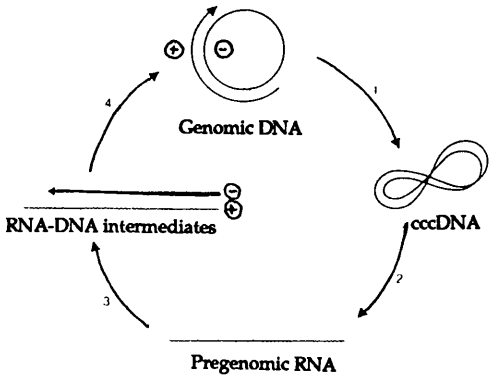

Figure 3. Replication Cycle of the Hepadnaviruses. From Ganem and Varmus (1987)

The virus attaches to the cell and, within an endosome, lysosomal enzymes may be responsible for stripping the surface/envelope protein from the virus, releasing it into the cytosol. The stripped virus particles are taken up by the nucleus where the DNA is uncoated and the protein attached to the 5' end of the minus strand and the ribonucleotides at the 5' end of the plus strand, are removed. A short terminal redundancy on the minus strand is also removed and the ends ligated together (Mason et al 1983). Enzymes responsible for these activities have not been identified. The plus strand is completed to form covalently closed circular DNA (ccc DNA), illustrated in figure 3, which is thought to act as the template for RNA synthesis

(Summers and Mason 1982). After infection, ccc-DNA is the main form of nucleic acid detected.

Several RNA transcripts of different sizes are synthesized by host RNA polymerase II but it is the 3.5 kb transcript which is used as the pregenome. It has several distinct features : it is longer than the viral genome and possesses a terminal redundancy of 130 to 270 nucleotides in length depending on the hepadnavirus. The terminal redundancy is derived through the initiation of transcription near the ORF-pre-C which is located upstream of the only known polyadenylation site in the genome. These signals for cleavage and polyadenylation are ignored during the first round of transcription but acknowledged on the second (Buscher et al 1985). Promoter elements found in the WHV genome and analogous to the major promoters of HBV, in particular the WHV E2 element (Di et al 1997). However, no analogous element has been found to the E1 element of HBV. They suggest that this may indicate a difference in the transcriptional regulation of WHV and HBV or that the genome may possess an E1 element which is not functional in their expression constructs. Alternatively transcription may be dependent on factors not found in the liver cell lines used. The DR1 sequence resides within the terminal redundancy region and minus strand DNA synthesis is initiated from here, by reverse transcription, at the 5' end. The end of DR2 is then thought to initiate plus strand synthesis using an oligomer of viral RNA as primer to generate the complete viral genome (Lien et al 1986).

Translation of the core/polymerase transcript occurs in the cytosol of the cell where core particles are assembled (Ou et al 1986). The rough endoplasmic reticulum is responsible for the assembly of the HBs proteins (Gumbiner and Kelly 1982, Patzer et al 1986, Bruss and Ganem 1991) which attach to the incoming core particles; the C-

terminus of the pre-S1 region can bind efficiently to core particles (Poisson et al 1997). The surface particles and mature virion are then exocytosed from the cell.

1.4 Viral Antigens

1.4.1 The Nucleocapsid

The nucleocapsid of HBV is composed of 180 core subunits which form an icosahedral structure with a diameter of 27nm as viewed under the electron microscope (Robinson et al 1976). Recently a three-dimensional map of the core protein using cryo-electronmicroscopy has revealed that the capsid is largely alpha-helical in structure (Bottcher et al 1997, Conway et al 1997). The subunits form dimers of alpha-helical hairpins which cluster on the surface of the shell to form 4 long radial alpha helices, giving a spiked appearance. The HBcAg, present on the surface of the nucleocapsid, is a basic protein with a molecular weight of 21kd (Gerlich et al 1982) which exists as two antigenically and morphologically distinct forms, HBcAg and HBeAg. Although HBcAg and HBeAg are serologically distinct they display substantial amino acid homology. ORF-C consists of 212 or 214 codons depending on the subtype of the virus (Pasek et al 1979, Tiollais et al 1981) and includes the pre-C region. The protein P21 is encoded from the second initiation ATG codon in the ORF-C and is not proteolytically processed. Biosynthesis of the HBeAg is initiated at the 5' ATG upstream of the core ATG. The resulting protein therefore has a pre-core signal sequence at its N-terminus which directs the protein to the ER where it undergoes proteolytic processing events, as outlined in figure 4.

The first 19 amino acids of the 29 amino acid pre-core sequence on the 25kd precursor are initially cleaved leaving a 10 amino acid extension at the N-terminus.

This short sequence was important in preventing the HBeAg forming up into particles as first suggested by Schlicht and Wasenauer (1991) who substituted this region with the signal peptide of an influenza haemagglutinin protein. The resulting product assembled into particles and displayed both HBc and HBe antigenicity.

The amino acids of the non-cleaved pre-core region contain a cysteine residue which forms an intramolecular disulphide bond with amino acid 61 which is also a cysteine residue (Nassal and Rieger 1993). In the absence of this bond a protein monomer of reduced HBe-antigenicity is produced. HBe dimers can also form by amino acid 61 forming an intermolecular disulphide bond with a second molecule which will now exhibit HBe and HBc antigenicity. Particle formation is prevented by the presence of a tryptophan-leucine-tryptophan hydrophobic tripeptide (Wasenauer et al 1992,1993). The 10 amino acid sequence is important for the quarternary structure and antigenicity of HBeAg.

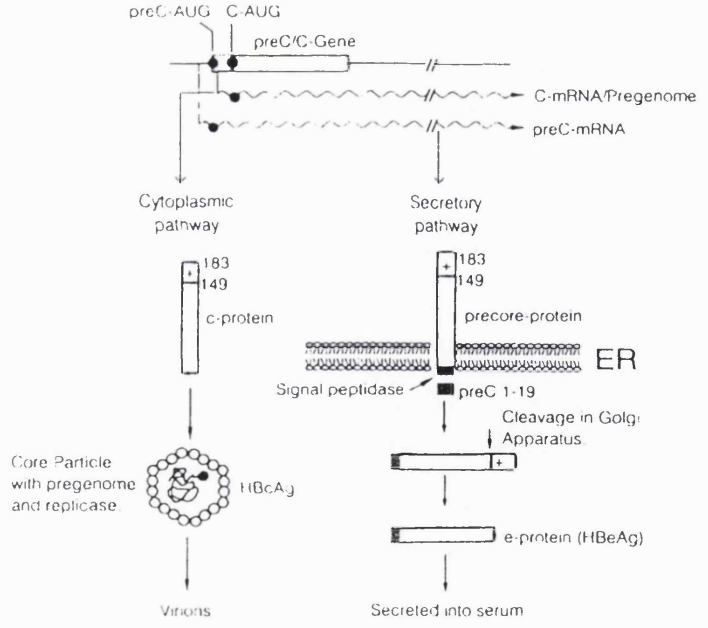

Figure 4. Biosynthesis pathways of ORF-C gene products. From Schlict, Wasenauer and Kock (1993)

Further processing occurs in the post-Golgi compartment where the carboxy terminal 34 basic amino acids are removed. In the HBcAg these 34 amino acids contain four clusters of arginine residues which are thought to act as a nucleic acid binding domain (Eckhardt et al 1991). In addition it has been found that deletion of the last 10, 25 or 39 amino acids at the C-terminus decreases the rate of HBeAg secretion which was totally abolished by deletion of the last 60 amino acids (Carlier et al 1995). HBeAg is then secreted and circulates as an 18kd polypeptide or as dimers (Ou et al 1986).

The WHV genome has an ORF-C which contains two in-phase ATG codons. Initiation from the first ATG is thought to produce a longer protein product which is post-translationally modified. Hantz et al (1983) found that crossreactivity existed between HBeAg and sera from chronically infected woodchucks. Recently Carlier et al (1994) described transient expression of the putative WHeAg in human 293 cells. The protein produced banded at a molecular weight of 18kd which is in agreement with the expected molecular weight if WHeAg follows the same processing pathway as HBeAg.

There are two potential sites of N-terminal glycosylation - Asn_{104}-Ile-Thr and Asn_{119}-Asp-Thr but only one form of glycosylated pre-core/core antigen, a 24kd protein. The pre-core/core antigen also contains an arginine rich domain and its amino acid homology with HBeAg is 79%. The signal peptide regions of the two viruses have 76% homology. It is thought that the pre-core/core protein is therefore processed in much the same way as HBeAg to produce the mature 18kd WHeAg. The cleavage at the C-terminus of the pre-core/core protein probably occurs at the HTVI-RRR sequence which is strikingly similar to the TTVV-RRR sequence in HBeAg (Takahashi et al 1983). It may be that the maturation pathway of 'e' antigen

is conserved amongst species infected with hepadnaviridae and if so it would provide another useful tool to study the course of infection.

Initiation from the second ATG in the ORF-C leads to the production of core protein (WHcAg) which forms particles. This mirrors what is observed with HBcAg in humans; HBcAg forms particles predominantly in the nucleus whereas WHcAg accumulates predominantly in the cytoplasm. 77% homology exists between WHcAg and HBcAg, illustrated in table 1 (Galibert et al 1982) and a significant antigenic crossreactivity is exhibited by WHcAg in commercial assay kits (Hantz et al 1983).

	GENE LENGTH (bp)	**NUCLEOTIDES**		**POLYPEPTIDE (aa)**	**AMINO ACIDS**	
		No.Identical	% homology		No.Identical	% homology
HBc	549	363	66	183	131	73
WHc	564			188		

Table 1. Illustrates the homology between the human and woodchuck core genes and their protein product (Galibert et al 1982)

The first demonstration that HBcAg induces protection against disease was by Iwarson et al (1985) and Murray et al (1987). WHcAg has been used to vaccinate woodchucks (Roos et al 1989, Schodel et al 1993) in order to boost intercellular T cell help. Roos et al (1989) immunized two woodchucks with 100ug recombinant WHcAg subcutaneously (s.c.) four times in four sites with Freunds incomplete adjuvant (IFA) over a period of 70 days. 10 days after the last immunization

woodchucks were challenged with WHV-positive serum and were found to be protected against infection. In addition the animals had developed high anti-WHc titres with no signs of WHV infection. In another study by Schoedel et al (1993) two groups of six woodchucks were immunized s.c. with 2 doses of either 50ug WHcAg or HBcAg, the first with CFA and the second IFA with a 12 day interval between doses. The animals were then challenged 10 days after the last dose was administered and all 6 immunized with WHcAg were protected against WHV infection compared to four out of the six immunized with HBcAg. Again high serum Ab titres against WHcAg or HBcAg were produced with no evidence of WHV infection after challenge.

1.4.2 The Surface Antigens

There are three surface antigens coded for by the ORF-S which contains three initiation sites and leads to the formation of three different surface proteins and their glycosylated forms, namely P24 and its glycosylated form gp27, P33 gp36 and P39 gp42. P24 corresponds to the s-gene and is a small protein (SHBS). P33 is the middle protein (MHBS) representing the product of the pre-S2 and s-gene and its glycosylated form, gp36, is thought to be required for virion secretion (Mehta et al 1997). Lastly P39, the large protein (LHBS), is derived from the pre-S1, pre-S2 and s-gene. The LHBS, the MHBS and the SHBS proteins are all found in the mature virion with the SHBS protein dominating in the 20nm subviral particles. The membrane contains host-derived lipids consisting of phosphatidyl choline phospholipid, free and esterified cholesterol and a small amount of triglycerides (Gavilanes et al 1982). Each subviral particle is composed of approximately 100 polypeptide monomers which are crosslinked by disulphide bonds. This particle is highly immunogenic. It has been found that in the majority of patients who recover

from acute infection neutralising Abs directed against an S epitope are present (Waters et al 1986). This immunogenicity can be decreased by reduction and alkylation of these -SH bonds (Vyas et al 1972, Imai et al 1974).

The pre-S components are not necessary for either construction or secretion of these subviral particles (Liu et al 1982, Laub et al 1983, Persing et al 1985). The particles self-assemble intracellularly in the endoplasmic reticulum (ER) and transport out of the cell occurs via the constitutive pathway through the Golgi apparatus (Gumbiner and Kelly 1982). The release of these particles is a characteristic feature of HBV infection and other hepadnaviral infections.

A number of subtypes of HBV have been described serologically which all have a common antigenic determinant designated the 'a' determinant. The four major subtypes are adw, adr, ayw and ayr which allow epidemiological patterns of infection to be traced and followed. In addition to these four subtypes there are several subspecificities assigned to the w determinant - adw2, adw4, ayw1, ayw2, ayw3, ayw4, $adrq^+$, $adrq^-$ and ayr (Norder et al 1992/93).

Crossreactivity does exist between HBsAg and WHsAg as evidenced by passive haemagglutination between the two antigens (Werner et al 1979). Commercial kits have also been used to detect WHsAg using cross-reacting sera to HBsAg in radioimmunoassay (RIA) or enzyme immunoassay (EIA) kits. WHsAg was detected in both Ausria II and Auszyme II kits both manufactured by Abbott Laboratories (Millman et al 1982, Hantz et al 1983). The RIA readily detects WHsAg which in infected animals is present in high titres. Anti-WHs can also be detected using RIA and EIA kits from Abbott (Millman et al 1988) although the sensitivity of these tests

is variable. This is consistent with the 74% nucleotide sequence homology found between human and woodchuck surface antigen genes (Galibert et al 1981).

The pattern of WHV infection of a woodchuck closely resembles that of HBV infection of humans. Infected woodchucks have circulating particulate forms of WHsAg in the blood which are predominantly subviral, spherical and filamentous particles composed of polypeptides which are also found on the surface of the virion. WHsAg has been used for vaccination and this strategy has been found to be successful in protecting chimpanzees from HBV infection (Cote et al 1986). In a more recent study using a different approach 7 chronically infected woodchucks were immunised with WHsAg along with a peptide named FIS (covering an amino acid region of 106-118 from sperm whale myoglobin) which is known to stimulate T helper cells (Hervas-Stubbs et al 1997). As a result all 7 animals produced high anti-WHs titres resulting in severe liver disease in one animal and death, due to the development of fulminant hepatitis, in another. As in humans antibodies to WHsAg may be protective; some doubt exists about the reliability of WHsAg as a diagnostic or predictive marker as it does not always appear after infection.

1.4.3 The Polymerase and X Antigen

The ORF-P protein product(s) is not well characterized and existing evidence that the polymerase enzyme is coded for by this gene is circumstantial. The gene itself is 834-845 codons in length and shares sequence homology with retroviral polymerase genes (Toh et al 1983). ORF-P overlaps the core gene at its distal end giving rise to the suggestion that a core-pol mRNA is produced from which a core-pol fusion protein is translated. This protein is then subjected to proteolytic processing to yield the mature polymerase protein. ORF-P however, differs from its retroviral equivalent because it

does not possess domains for an integrase and a protease but does possess a primase domain which is lacking in retroviruses. The polymerase protein was first detected as two high molecular weight bands, one of 90kd and the other of 70kd (Barand and Laub 1988). A 70kd band was also detected by Mach et al 1988. However, the 90kd protein is thought to predominate in the virions (Bartenschlager and Schaller 1992) and to be phosphorylated at two sites (Ayola et al 1993).

ORF-X is a 154 amino acid coding region although its product(s) structure and function in viral replication is unknown. There is no evidence to suggest that it is a structural component of virions. It is thought that it may be involved in transactivating gene transcription (Twu and Schloemer 1987, Arii et al 1992), a biochemical activity e.g protein kinase (Wu et al 1990), protease inhibitor (Arii et al 1992), ribo/deoxy ATPase (Medina et al 1994) or as a tumour promoter (Terradillos et al 1997). It was suggested by Hohne et al (1990) that it is involved in tumour suppression. They found that the retention of the tumour suppressor protein p53 in the cytoplasm of FMH 202, an immortalized hepatocyte cell line, was associated with the X protein.

WHV also encodes a viral polymerase enzyme which is involved in replication of the virion. The X gene is also found in WHV and overlaps the polymerase and pre-core genes and two new putative ORFs termed ORF-5 and ORF-6 whose importance is not known. A study by Chen et al (1993) found that neither ORF-5 nor ORF-6 codes for a protein or is required for viral replication. They may be a remnant of the ancestral virus; multiple overlapping ORFs are found in retroviruses and as both hepadnaviruses and retroviruses share their origins (Miller et al 1986, Robinson et al 1987) this may account for their presence. Both groups suggest that the WHV X

gene is important for viral replication in vivo in its natural host. However, whether it acts as a transactivator in the course of infection has not been determined.

1.5 Clinical Outcomes of Infection

1.5.1 HBV Infection

Infection with HBV can result in an acute self-limiting infection or alternatively in a persistent chronic carriage of the virus. A period of between six weeks and six months is considered to be the normal incubation time of the virus. Acute hepatitis is transient and often characterized by a rise in serum transaminases; patients are usually asymptomatic. In some cases the patient may suffer from malaise leading to jaundice, hepatomegaly (detected in 70% of patients), splenomegaly (detected in 20% of patients) and arthropathy of the small joints. These clinical features are accompanied by abnormal liver function tests. A patient suffering from fulminant hepatitis B deteriorates rapidly with complete destruction of the liver. A milder initial course may occur in a patient suffering from chronic hepatitis and the patient may then present with the complications of cirrhosis or primary hepatocellular carcinoma before infection is detected. The degree of liver injury which occurs in patients suffering from chronic hepatitis B varies depending upon the stage of the disease. In an asymptomatic carrier there is no liver injury, in a patient with mild to severe chronic hepatitis the liver sustains more damage. In addition HBV has been associated with other extrahepatic clinical features, for example polyarteritis nodosa, membranous glomerulonephritis and acrodermatitis as well as other documented manifestations due to the formation of immune complexes.

1.5.1.1 Acute Hepatitis B Virus Infection

The appearance of HBsAg accompanies the onset of acute hepatitis B and is the main diagnostic and screening marker of infection. HBsAg concentrations continue to rise along with serum alanine aminotransferase (ALT) levels (figure 5). A high IgM-anti-HBc level indicates acute phase disease. At around the same time that the elevation of HBsAg is documented, HBeAg and HBV DNA start to appear and the levels rise in the serum correlating with ongoing viral replication. Seroconversion from HBeAg to anti-HBe antibody precedes HBsAg clearance and together with the fall of HBV DNA, indicates cessation of replication and onset of recovery. HBsAg seroconversion to anti-HBs confirms recovery.

In patients with acute infection less than 1% progress to a fulminant course with death being the usual result. Of the few that survive a fulminant course progression to chronic disease is rare.

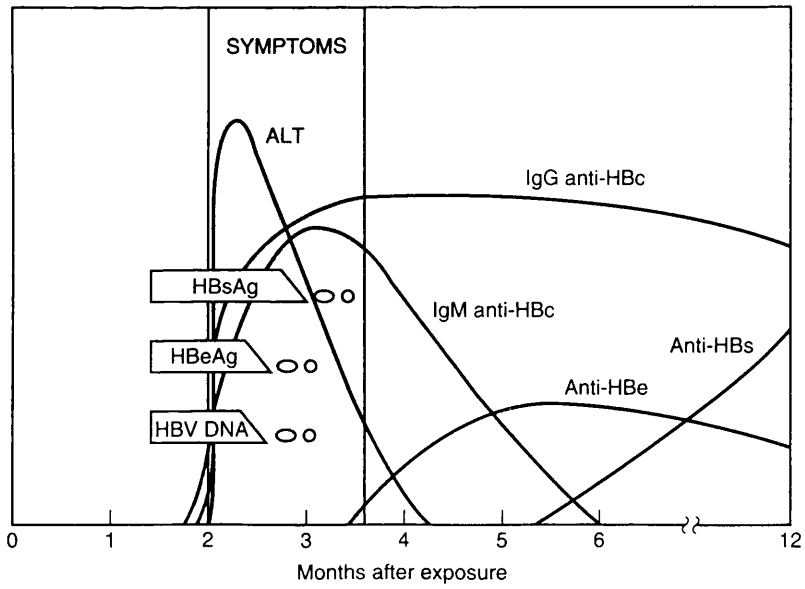

Figure 5. Serological Profile of the Markers of HBV during and after Acute Infection (Zuckerman and Thomas 1993).

1.5.1.2 Chronic Hepatitis B Virus Infection

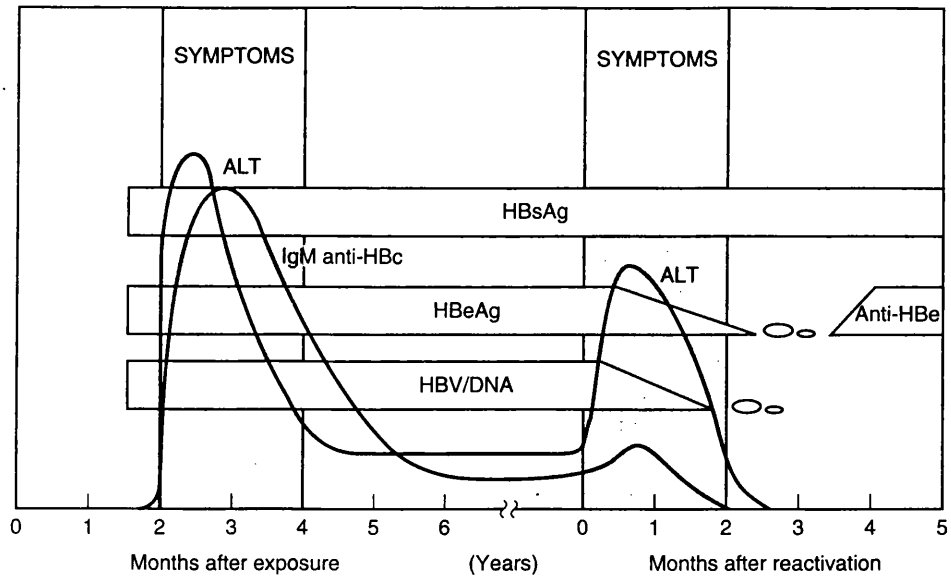

Figure 6. Profile of Serological Markers Showing Progression from Acute to Chronic Infection Followed by the Non-Replicative Phase (Zuckerman and Thomas 1993)

The persistence of HBsAg in the serum for a period of six months defines chronic infection. Of the 5-7% that become chronically infected 10-15% of these adults will spontaneously seroconvert to anti-HBs between a six month to 10 year period whilst remaining normal for liver function tests. 90% of perinatally infected babies and 50% of those infected within the first 2 years of life develop persistent infection with low probability of spontaneous seroconversion. During chronic infection HBV DNA and HBeAg levels are detectable whereas IgM-anti-HBc declines but total anti-HBc does not. ALT levels may be elevated indicating active disease (figure 6) which, if prolonged, may lead to cirrhosis and primary hepatocellular carcinoma (Hoofnagle et al 1987).

Chronic hepatitis has been classified morphologically according to liver biopsy as chronic persistent hepatitis (CPH), chronic active hepatitis (CAH) and chronic lobular hepatitis (CLH). CPH is described as a mild disease characterized by portal tract inflammation with infiltration of lymphocytes and other cells. CAH can be distinguished from CPH by the presence of lymphocytes surrounding the hepatocytes

lining the portal tract, the so-called piecemeal necrosis. CLH can be seen in CAH and CPH and is diagnosed by the presence of necrosis within the acini or lobules with minimal portal infiltration (Scheuer 1989, Desmet et al 1994).

Recently the histological classification has changed encompassing aetiology, grade and stage of disease (Hyteroglou et al 1995). It has been suggested that necroinflammatory activity be graded 0-3 ranging from no necroinflammatory activity to severe piecemeal necrosis and lobular activity with or without bridging necrosis. In addition it suggests that stage of fibrosis/cirrhosis be graded 0-4 ranging from no fibrosis to cirrhosis. This classification would enable clinicians to assess and evaluate the effects of therapy more accurately.

Patients may become symptomatic immediately after acute disease or some years later after an acute exacerbation, producing increased ALT levels. A small proportion of chronically infected patients (1-2% annually) seroconvert from being HBeAg positive to being anti-HBe positive, although HBsAg persists. A proportion of chronic HBV carriers seroconvert to anti-HBe and eventually lose their HBsAg and become anti-HBs positive. A proportion of patients remain HBsAg positive and have integrated HBV DNA and remain at risk of developing HCC and cirrhosis. Some, who become anti-HBe positive have ongoing progressive liver disease. Some patients with point mutations in the pre-core region of the HBV genome may have no HBeAg and detectable HBV DNA (Carman et al 1989). The cause of these patients liver disease is unclear; on the disappearance of HBV DNA from the serum they become carriers of the mutant virus. Nucleotide 1896 G to A was the most frequent mutation found in 90% of HBeAg negative carriers (Okamoto et al 1990). The pre-core mutant does arise in other patients during seroconversion but they are still able to clear the virus. The proportion of patients who spontaneously convert from replicative (HBV DNA

positive, HBeAg positive) to non-replicative (HBV DNA negative, HBeAg negative) chronic HBV infection is approximately 10-15% per year.

1.5.2 WHV Infection

The age at which WHV infection occurs appears to have a bearing on the outcome of infection. 30% of woodchucks that are 8 days old or younger when infected develop chronic WHV infection, whereas the proportion who develop chronic infection once they are 8 weeks old, drops to 19% (Tennant et al 1988). This situation is mirrored in man and it is clear that perinatal transmission of the WHV virus plays a role in the high prevalence of chronic infection observed in captured woodchucks. Kulonen et al (1988) have provided evidence for the vertical transmission of disease. 75% of fetal livers in the uterus of pregnant dams with disease, were found to be WHV-DNA positive with integration of WHV DNA into the chromosomal DNA of the liver.

1.5.2.1 Acute WHV Infection

Experimental infection of animals can result in differences in the time of onset of disease. It has been reported as early as 5 and 6 weeks post infection (p.i) (Ponzetto et al 1984, Tyler et al 1986) and as late as 12 weeks p.i. (Popper et al 1987). Experimental acute infection appears to be dependent on age at the time of infection with younger woodchucks being more susceptible, possibly due to an immature immune system. Although older woodchucks can be successfully infected, fewer adult woodchucks (10%) developed persistent infection following experimental infection, than newborn woodchucks (30%). In addition, of the 30% who became chronically infected 85% developed HCC within 3 years (Tennant et al 1988). Clearance of the

disease coincides with loss of WHV DNA and detection of anti-WHs (Millman et al 1984, Tyler et al 1986). In addition woodchucks can be infected with GSHV although the reverse is not true (Seeger et al 1987).

Infection with WHV can lead to acute and chronic infection. Acute disease, which is transient and self-limited, is characterized by focal hepatocellular necrosis. In the area surrounding the necrosis a range of cell types are found, notably mononuclear cells, sinusoidal cells, neutrophils and eosinophils which result in expansion of the portal tract (Popper et al 1981).

1.5.2.2 Chronic WHV Infection

Histological differences in the liver disease distinguish different forms of chronic disease; chronic active hepatitis (CAH) is witnessed more frequently than chronic persistent hepatitis (CPH) or minimal hepatitis (Frommel et al 1984, Michalak 1988, Abe et al 1988). The liver lesions in these animals show portal tract expansion due to mononuclear cell infiltration and occasionally portal and periportal fibrosis. The 'ground glass' cell which is seen in HBV infection is thought to be produced by an imbalance of HBsAg proteins which produces a reticulated or granular appearance of the cell. This phenomenon has been described in some woodchucks (Abe et al 1988) but it is not a consistent occurrence.

Apart from hepatocytes in the liver, WHV does have the ability to replicate in different cell types and organs; the lymphoid cells of the bone marrow, spleen, lymph nodes, thymus, pancreas, kidneys and ovaries have all shown evidence of the presence of WHV DNA (Korba et al 1987, Korba et al 1989, Korba et al 1990). In addition WHV DNA has been found in peripheral blood lymphocytes (PBL) of chronically

infected woodchucks (Korba et al 1986) and when stimulated with the mitogen lipopolysaccharide (LPS) the production of viral replicative DNA intermediates can be detected (Korba et al 1988). There is no evidence of comparable HBV replication in human PBLs.

In humans HBV antigens are expressed on the surface of hepatocytes during infection and are thought to play a crucial role in instigating the immune response to cause disease pathogenesis (Mondelli and Eddleston 1984). This situation is mirrored in the woodchuck whose hepatocytes display virus nucleocapsid and envelope polypeptides both in the acute and chronically infected animal (Michalak and Lin 1994). Whether liver damage is due to the same immune mechanisms remains to be determined.

1.6 The Immunology of HBV Infection

1.6.1 The Immune Response to Viral Antigens

It is thought that HBV is not directly cytopathic; instead the liver disease is due to the immune response of the host against viral antigens (Dudley et al 1972). Studies using transgenic mice expressing the HBV genome in kidney tubular epithelial cells have demonstrated that HBV gene products are not directly cytopathic for the hepatocyte (Chisari and Ferrari 1995). Even in mice that accumulate non-secretable HBsAg particles in the ER of the hepatocyte no destruction is observed (Chisari et al 1986). However, it is possible to destroy them using physiological amounts of IFN-gamma (Gilles et al 1992). In addition it has been shown that adoptive transfer of CD8+ MHC Class I restricted HBsAg-specific CTL to transgenic mice expressing the HBV envelope antigens on their hepatocytes induces acute liver disease and

interferon gamma production (Chisari et al 1990). Interleukin 12 (IL-12) is a heterodimeric cytokine secreted by antigen presenting cells (APCs) which increases IFN-γ production, NK cell activity and favours Th1 cell development. Treatment of HBV transgenic mice with IL-12 results in the disappearance of HBV replicative intermediates form the liver and kidney tissues (Cavanaugh et al 1997). They also showed that IFN-γ, IFN-α, IFN-β and TNF-α are produced in response to IL-12 treatment and that the principle mediator of this anti-viral effect was IFN-γ, which in turn causes the upregulation of high affinity IL-12 receptor expression (Gollob et al 1997). This system forms a positive feedback loop (Nauomov and Roussol 1997).

There are two antigen processing pathways known as the Class I pathway which processes endogenous antigen, such as viral antigens, and the Class II pathway which processes exogenous antigen. Exogenous antigen bound to its receptor in clathirin coated pits on the cell membrane is internalized by invagination of the membrane. The clathirin is then removed to form a vesicle and transferred to the early endosome. Part of this compartment forms the late endosome which moves to the trans-Golgi network to bind MHC Class II in a specialized compartment termed the 'compartment for peptide loading '(CPL) (Tulp et al 1994) before returning to the surface. In order to prevent the binding of Class II to peptides in the ER, Class II is synthesized as a trimer of alpha, beta and invariant (Ii) chains. The Ii chain prevents binding of endogenous peptides to the MHC (Roche and Cresswell 1990, Lotteau et al 1990, Neefjes and Ploegh 1992). When the MHC Class II molecule exits the ER, the Ii chain is dismantled and the MHC-antigen complex moves to the cell surface.

The Class I pathway processes endogenous antigen in the cytoplasm. The MHC Class I molecules are synthesized in the ribosome of the ER and transported via the lumen of the ER to the outside. The molecules are composed of two chains termed the alpha

chain and beta-2 microglobulin (Bjorkman et al 1987). It is thought that the cytoplasmic viral antigens are degraded into peptides by a proteasome which contains multiple catalytic sites (Brown et al 1991). This may aid the production of nine amino acid peptides (Rudensky et al 1991) which are delivered to transporters residing in the ER membrane. These peptides associate with the MHC molecules where conformational changes may be induced which allows export of the complex to the surface (Townsend et al 1989, Monaco 1992). An exogenous viral antigen will be internalized by an antigen presenting cell (APC) e.g. macrophage, dendritic cell, B cell, where they are degraded by enzymes and processed. The peptides are presented on the surface of the cell in conjunction with a major histocompatibility complex (MHC) gene product (either Class I or Class II); $CD4^+$ T helper cells possess a T cell receptor (TCR) which recognizes antigens bound to the MHC Class II whereas $CD8^+$ cytotoxic T cells recognise antigens bound to MHC Class I, a phenomenon called MHC restriction (Zinkernagel and Doherty 1974).

The T cell receptor (TCR) is a complex of a CD3 heterodimer and Ti chain (Davis and Bjorkman 1988) which together with the MHC and antigen form a ternary complex which provides a signal for T cell activation. The TCR may provide a signal via a tyrosine kinase enzyme termed $p56^{lck}$. In addition to the signal from the ternary complex costimulatory signals are required in order for T cell activation to proceed (Springer et al 1987, Weiss and Imbroden 1987). One important example of these co-stimulatory molecule interactions is the CD28 molecule, shown in figure 7, and its physiological ligand B7. CD28 is a 44kDa adhesion receptor found on the surface of T cell subsets which binds to B7 and stimulates IL-2 production which facilitates proliferation of T cells (Linsley et al 1991). These signals are provided by accessory cells and in their absence Th cell anergy or unresponsiveness to antigen may result

(Mueller et al 1989, Schwartz 1990). This may play a role in the carrier state of disease in hepatitis.

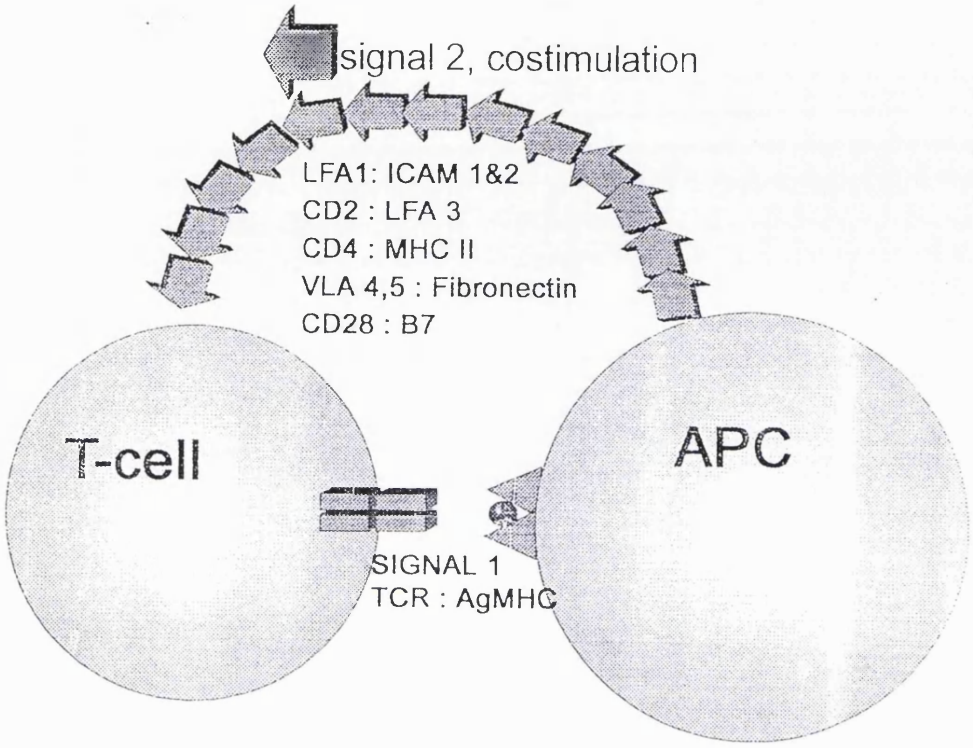

Figure 7. Simplified Diagram of the Signals Involved in the Initiatory Step of the Immune Response (Heitman 1994)

Unprocessed antigen possesses B cell determinants which are recognized by Ig receptors on the B cell surface. Abs are produced by plasma cells as a result of this recognition. The B cell response is regulated by cytokines released by activated T cells. T cells can be subdivided into subsets characterized by their pattern of cytokine secretion. Postthymic T cells which have not encountered antigen are referred to as naive T cells and will only secrete IL-2 on first stimulation (Croft et al 1992). They have the potential to develop into two different populations of primary effector cells termed T helper 1 (Th1) or T helper 2 (Th2) subsets. Th1 cells secrete IL-2, IFN-

gamma, lymphotoxin and granulocyte-macrophage colony stimulating factor (GM-CSF). Th2 cells secrete IL-4, IL-5, IL-6, IL-10, IL-13 and GM-CSF (Mossmann and Coffman 1986). Cells which can secrete a full range of cytokines are referred to as Th0. In addition there are T memory cells which can be Th0, Th1 or Th2 (Swain 1993) as schematically shown in figure 8.

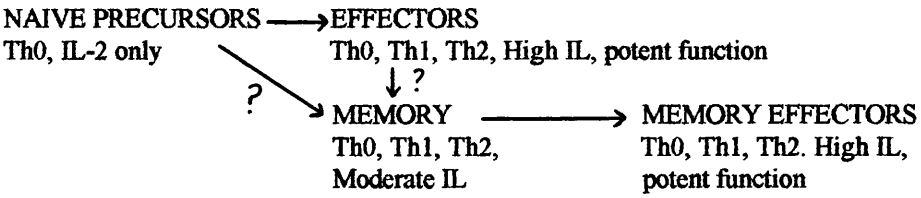

Figure 8. Schematic respresentation of Th cell development (Swain 1993).

1.6.2. B Cell Mediated Immunity

Anti-preS antibodies (Abs) may appear early after infection (Alberti et al 1990) and may be detected at clinical onset of disease. In contrast the second phase of anti-HBs appears after the late response. For this reason the pre-S Abs are used as a marker of infection whereas the S Abs are used to confirm recovery from infection.

Anti-HBs protects against infection with HBV and the current vaccines, which are safe and effective, and contain the major envelope antigen alone. A high proportion of subjects respond although this ranges from high responders to non-responders. Approximately 5% of those vaccinated are low or non-responsive after three doses of the vaccine, including dialysis patients (Dienstag et al 1984). An association has been suggested between MHC and non-responsiveness to vaccine; 45% (Craven et al 1986) and 48% (Weissman and Tsuchiyose 1988) of hyporesponsive patients to Hepatavax B (Merck, Sharpe and Dohme) were found to be HLA-DR7 positive.

In the majority of patients HBeAg disappearance and anti-HBe appearance has been used as a marker of cessation of viral replication as this occurs concurrently with histological remission of liver disease (Realdi et al 1980). A mother infected with wild type who transmits the infection to her baby will cause the latter to develop a persistent infection (Stevens et al 1975). This is thought to be due to a tolerant state induced in utero by the transmission of HBeAg transplacentally (Milich, Jones et al 1990). There are two antigenic epitopes on the HBeAg - HBe1, which is linear, and HBe2, which is conformational and may be masked in the assembled core particle (Imai et al 1982, Salfeld et al 1989). HBV variants exist which possess an aberrant stop codon preventing pre-core region transcription (Carman et al 1989) and thus HBeAg formation. It has been postulated that these patients are initially infected with the wild type virus and, under immune pressure, the virus mutates, affecting HBeAg formation in an attempt to escape immune surveillance. This is now thought to be a natural progression of the disease state.

In mice the particulate HBcAg is a T cell independent and dependent antigen which has proved to be very immunogenic (Milich and McLachlan 1986). In humans it is conceivable that HBcAg can circumvent the need for T cell help by binding directly to B cell surface receptors and producing a vigorous Ab response resulting in a high IgM anti-HBc titre characteristic of HBV infection. IgM anti-HBc Abs are produced early during the course of acute infection and are used as a diagnostic marker. IgM and IgG anti-HBc can persist in the serum of an HBV infected individual for a lengthy period of time albeit with a gradual reduction in titre (Hoofnagle et al 1973).

Although antibodies to the non-structural polymerase (Chang et al 1989, Yuki et al 1990) and X (Stemler et al 1990, Levrevo et al 1991) proteins have been detected they are not thought to be useful tools for diagnostic purposes.

1.6.3 T Cell Mediated Immunity

The non-particulate protein HBeAg, derived from the pre-core/core region, is a T cell dependent antigen and there is a high degree of crossreactivity with HBcAg at the T cell level (Milich et al 1988). It has been suggested that the high titre of anti-HBc found in patients may be due to this crossreactivity, caused by the expansion of a large Th cell population which stimulate more anti-HBc producing B cells (Milich et al 1988).

The importance of the MHC Class II restricted T cell response to viral antigens on the outcome of infection is highlighted in a number of studies. In Qatar an excess of DR7 and a deficiency of DR2 alleles was associated with susceptibility to persistent infection (Almarri and Batchelor 1994). DRB1*1302 has been linked to recovery from HBV infection in both adults and children in the Gambia (Thursz et al 1995) and Caucasian patients (Hohler et al 1997). The response was MHC Class II restricted (HLA-DPw4) in another study (Celis et al 1988) where the existence of a T cell immunodominant epitope was situated at the amino terminus of the major envelope protein which is 10-30 amino acids in length. Experiments have been carried out in the murine system that show that in strains that do not respond to the major envelope protein a highly immunogenic response is elicited when immunization with the pre-S proteins is carried out (Milich et al 1985,1986). These experiments show that the responses to pre-S and S are under different Ir gene control and the importance of the pre-S response is not known. Pre-S1 and pre-S2 may be involved in viral attachment (Neurath et al 1986) and entry into the hepatocyte.

Patients who have a self-limited acute infection display a strong HLA Class II restricted peripheral blood T cell response to HBcAg and HBeAg (Ferrari et al 1990,

Jung et al 1991) often associated with a transient clearance of HBsAg from the serum. This $CD4^+$ mediated T cell response is significantly lower or absent in chronically infected patients who fail to spontaneously clear the virus. This pattern is mirrored by CTLs in the acute and chronic case suggesting that $CD8^+$ T cells are dependent on $CD4^+$ nucleocapsid specific helper T cells for the elimination of infected cells and in the pathogenesis of HBV.

Studies have been carried out to identify immunodominant epitopes on the nucleocapsid protein. Ferrari et al (1991) used a series of synthetic peptides to stimulate cell lines from acute patients and found a strong proliferative response to peptides covering nucleocapsid amino acids 1-20, 50-69 and 117-131. In addition responses were generated to peptides 19-39 and 64-83. These peptides are also generated in vivo since T cell lines produced by stimulation with these peptides respond to whole HBcAg. Identification of the HLA restriction elements of these peptides is yet to be determined. Table 2 illustrates the homology between woodchuck and HBV core at these epitopes.

Peptide	**WHV Core Sequence**	**Length/aa**	**% Homology**
1-20	MDIDPYKEFGSSYQLLNFLP	20	75
19-39	LPLDFFPDLNALVDTATALYE	21	62
50-69	PHHTAIRQALVCWDELTKLI	20	65
64-83	ELTKLIAWTSEQVRTIIVNH	20	35
117-131	FGVWIRTPAPYRPPN	15	87

Table 2. Comparison of the Same Sequences of HBV and WHV core based on the known T cell epitopes found in humans by Ferrari et al (1991).

Jung et al (1995) found that the fine specificity of the $CD4^+$ T cell response to nucleocapsid peptides was similar in both acute and chronic patients. In addition they found that repeated testing to the peptides during acute infection increased the percentage of patients who responded. Amino acid regions 1-25 and 61-85 were identified as the predominant CD4+ T cell recognition site regardless of HLA haplotype. These residues are thought to be located at the tip of the hairpin forming the alpha-helical spike on the surface of the core shell (Bottcher et al 1997, Conway et al 1997). Sequences 21-45, 41-65 and 81-105 were also identified as important regions. In mice, residues 120-140 of HBcAg was found to produce a strong response in $H\text{-}2^s$ or $H\text{-}2^b$ mice whereas $H\text{-}2^f$ and $H\text{-}2^q$ mice recognized residues 100-120 (Milich et al 1988). Due to developments in cell assay systems (Kreuzfelder et al 1996, Menne et al 1997) T cell proliferative assays became possible in woodchucks. In a recent study T cell proliferative responses to WHcAg were assessed in 8 acutely infected woodchucks (Menne et al 1997). The peptide 97-110 produced strong proliferative responses in all 8 animals compared to other peptides which produced lower stimulation indices in fewer animals. Furthermore, animals immunized with this immunodominant epitope 97-110, were protected from infection when challenged but did not produce any antibody. Animals immunized with recombinant WHcAg were also protected from infection but produced a strong anti-WHc response. This group suggests that protection is more dependent on T cell responses than humoral responses.

In the mouse, HBcAg specific T helper cells can also assist the production of anti-envelope antibodies by providing help to B cells (Milich et al 1987). Intermolecular T cell help may also occur in humans as Ferrari et al (1990) have found that a proliferative response to nucleocapsid antigens in acute patients is associated with clearance of HBsAg. Immunization of chimpanzees with HBcAg confers protection

against HBV infection (Iwarson et al 1985, Murray et al 1987) and this is independent of anti-HBs. They suggested that it is not anti-HBc which is responsible for the protection evidenced, although Ab may modulate the course of infection (Pignatelli et al 1987), but the cell-mediated arm of the immune system. Fusion proteins of HBcAg carrying an immunodominant epitope of HBsAg can form particles with HBc antigenicity which can induce a T cell proliferative response to HBcAg and HBsAg (Shiau and Murray 1997).

More recent work using CTL lines, derived from the peripheral blood of acute HBV patients, has identified a small peptide corresponding to amino acid residues 11-27 which can induce CTL activity in an HLA-A2 positive individual (Bertoletti et al 1991, Penna et al 1991). This response could not be detected in chronically infected patients. Furthermore, the optimal cytotoxic T cell epitope was mapped to residues 18-27 (Bertoletti et al 1993) which contains a leucine at position 2 and valine at position 10, thought to anchor the peptide into the HLA-A2 binding groove (Bjorkman et al 1987). It has also been documented that pre-S2 specific $CD8^+$ T cell clones from CAH patients display cytolytic activity against autologous HBsAg presenting target cells (Barnaba et al 1989). The presence of CTLs to both nucleocapsid and envelope proteins has been demonstrated in both acute (Nayersina et al 1993) and chronically infected patients (Van Hecke et al 1994) which may indicate that different viral antigens can act as targets for CTL mediated lysis. Recently a new mutational domain was found in the HBsAg isolated from the livers of chronic carriers. It was found at amino acid positions 40 and 47 which coincides with an MHC class I-restricted T cell epitope (Tai et al 1997). The importance of this domain in evading a CTL response and establishing a persistent infection remains to be determined. CTLs to the RNAase H and reverse transcriptase domains of the polymerase protein have also been detected in acute HBV patients but not chronically

infected patients (Reherman et al 1995). The CTLs may play a dual role in HBV infection by i) causing disease pathogenesis through immune mediated lysis and ii) by regulating HBV gene expression via IFN-gamma and TNF-alpha as has been suggested by Guidotti et al (1994) from work on transgenic mice. The disparity between the acute and chronic immune response to nucleocapsid proteins suggests that these are the primary antigens involved in the pathogenesis of HBV infection and that this difference is responsible for the outcome of infection. One reason for this difference in disease pathogenicity may lie in the balance between Th1 and Th2 cells. Th1 cells contribute to cell mediated immunity i.e. CTL response, whereas Th2 cells are predominantly involved in the humoral response. Milich et al (1995) propose that a Th1 response in combination with the MHC restriction element predominates in acute patients causing clearance of the virus and Th2 cells predominate in chronic patients who mount an inadequate Ab response and allow the virus to persist. Furthermore, he suggests that HBcAg preferentially, although not exclusively, encourages Th1 cell development and that HBeAg preferentially, although not exclusively, encourages Th0 or Th2 cell development (Milich et al 1997). In other words Th cell phenotype can be influenced by antigen structure as well as cytokine treatment. A recent study by Nakamura et al (1997) has found that RT-PCR can be used to detect IFN-γ and tumour necrosis factor-alpha, in addition to the CD3ε subunit, in woodchuck PBMCs and liver cells. They speculate that differential RNA expression of these cytokines can indicate whether there is a defect in the Th1 response which may indicate the risk of progression to chronicity.

1.7 Hepatocellular Carcinoma (HCC)

The association between primary HCC and persistent HBV infection as the aetiological agent has been documented for populations in Eastern Asia and sub-

Saharan Africa where the virus has a higher prevalence. This correlation between persistent infection and HCC has also been detected in other mammals, namely the woodchuck (Summers et al 1978) and ground squirrel (Marion et al 1983) which suggests that hepadnaviral infection is a significant factor contributing to HCC development. The oncogenic potential of WHV in woodchucks is greater than it is for HBV in humans but the progression from chronic hepatitis to hepatoma does not occur concurrently with liver cirrhosis in woodchucks as it does in humans (Popper et al 1981, Snyder et al 1982). HCC is associated with several pathological features outlined in table 3.

The liver enzyme serum alanine aminotransferase (ALT) has been used as a marker of acute liver damage in woodchucks (Korba et al 1989) in addition to the enzyme gamma glutamyltranspeptidase (GGT) which, although not specific for HCC, is a useful marker of tumour development and rises linearly with tumour growth. Alpha-fetoprotein (AFP) is usually present at very low levels in the serum of healthy non-pregnant woodchucks but is elevated in animals with HCC; it requires a species-specific test for its detection.

Feature	Woodchuck	Man
Trabecular or pseudoacinar HCC	+	+
Eosinophilic globules in tumour cells	+	+
Hyperplastic nodules	+	0
Clear cell foci	+	0
Cirrhosis	0	+
Portal and periportal inflammation	+	0
Inflammation within tumour	+	0
Haematopoietic cells	+	0
Metastasis	0	+
Association with serum markers of viral		
Replication e.g. sAg,eAg, viral DNA	+	0
Integrated viral DNA in tumour cells	+	+

Table 3. Pathological features associated with HCC (+ present, 0 absent)

On examination of the livers of chronic animals WHV-DNA integration has been found prior to transformation of the tissue into tumourous liver nodules (Ogston et al 1982, Rogler et al 1984, Kaneko et al 1986). This integration is thought to be the initiating step in the oncogenic process. Secondary events have been implicated in HCC due to the extensive rearrangement observed of the integrated WHV-DNA in advanced HCC which is absent during the early stages of the carrier state. The *myc* family of cellular proto-oncogenes have been associated with the development of liver tumours. The insertional activation of *c-myc* has been associated with 10% of

woodchuck HCCs whereas a stronger association (40%) was observed with *n-myc* (Moroy et al 1989, Fourel et al 1991). In man however no such pattern has been discerned between the *myc* family and HCC.

1.8 Antiviral Therapy

All forms of chronic viral hepatitis are the target for any antiviral strategy delivering a permanent suppression of viral replication as measured by serum HBV DNA levels and HBeAg levels and elimination of infected hepatocytes as measured by serum aminotransferase levels and examination of liver histology. There is no effective therapy for the treatment of HBV infection. Current treatments such as interferon are expensive and are only partially effective, particularly in treating chronic adult carriers from countries of high endemicity. Among those patients who develop primary HCC the death rate exceeds 90% despite surgery and chemotherapy. Numerous drug trials have been undertaken in an attempt to improve the prognosis of HCC but as yet, a safe and effective drug remains elusive.

The purine nucleoside, adenine arabinoside (Ara-A) and its monophosphate derivative (Ara-AMP), were under study in the early 1980s (Bassendine et al 1981, Hoofnagle et al 1984, Perillo et al 1985, Trepo et al 1986) because of their potent inhibitory action on DNA polymerase activity. However, due to a limited efficacy and neuromuscular toxic side effects both drugs were withdrawn from use. Ponzetto et al (1990) and Fiume et al (1995) report that doses of Ara-AMP or ACV-monophosphate used to treat woodchucks can be reduced if coupled with lactosaminated human serum albumin (L-HSA) or poly-L-lysine respectively as evidenced by a fall in WHV-DNA levels.

Non-cyclic guanosine analogues, such as Acyclovir and its oral prodrug 6-deoxyacyclovir, were tried because they were considered safer but were proven ineffective in clinical trials (Weller et al 1986, Alexander et al 1987). Acyclovir (ACV) is one of the antiviral agents preclinically tested on woodchucks; serum DNA polymerase activity was found to be reduced after treatment (Hantz et al 1984). In a more recent study WHV DNA was cleared from the serum and replaced by virion-like particles containing minus strand WHV DNA after treatment with ACV (Tencza and Newbold 1997). Minus strand DNAs were also found in infected hepatocytes thought to be truncated by ACV.

Drugs such as suramin and zidovudine act by inhibiting reverse transcriptase whilst others modify the immune response e.g. levamisole, corticosteroids and IL-2. Levamisole increased the level of soluble IL-2 receptors in one study where it was used in conjunction with IFN but did not result in increased effectiveness in comparison to IFN treatment alone (Ruiz-Moreno et al 1993). IFN combined with IL-2 again showed no enhancement of the immune response and proved to be extremely toxic (Bruch et al 1993).

Interferon (alpha and beta) has proved to be the most widely used anti-viral and immunomodulatory agent and acts by stimulation of macrophages, enhancement of NK activity and increased MHC Class I expression as well as stimulating TNF and IL-2 production and acting directly as an anti-viral. An HBeAg/anti-HBe seroconversion rate as high as 50% has been produced using lymphoblastoid alpha interferon (Mazzella et al 1988). Recombinant alpha interferon has been employed in several clinical trials and can induce remission in 40% of selected HBsAg carriers (McDonald et al 1987, Hoofnagle et al 1988, Dusheiko et al 1988, Lok et al 1988, Perez et al 1988).

The use of recombinant interferon alpha-2b in a large clinical trial (Perillo et al 1990) showed that 37% of patients seroconverted and lost HBV DNA from the serum. The great majority of patients who respond to IFN sustain these responses; 5% report back with reactivated infection. The rate of seroconversion in another study was found to be 20% when IFN-alpha-2b was administered to HBeAg positive chronic hepatitis B patients (Wong et al 1993). IFN does have side effects, namely fever, chills, myalgia and fatigue. In addition autoimmune hepatitis is known to be exacerbated during IFN therapy (Papo et al 1992).The long term efficacy of this drug in delaying the onset of HCC remains to be determined. A recent study by Nauomov and Roussol (1997) found that in 33 patients undergoing IFN-α treatment 10 patients cleared HBV and had a 2.5 fold higher level of IL-12 in their serum during or after treatment compared to non-responders. It is thought that IFN-α increases expression of the IL-12 receptor β2 subunit and thereby amplifies Il-12 action.

The alternative to single agent therapy is a combination of two or more agents. One advantage of this treatment is in patients who do not respond to IFN-alpha alone, because they acquired the infection at birth, who may respond to the combined therapy. IL-12 is undergoing clinical trials as a monotherapy but possibilities exist for its use in combination therapy. Inhibition of Il-4 and IL-10 may also be required to switch on Th1 responses and increase the efficacy of IlL-12 treatment (Thomas and Waters 1997). The most successful regimen found so far, and one which produced a higher seroconversion rate, is pretreatment with prednisolone followed by IFN alpha therapy (Perillo et al 1988, Krogsgaard et al 1996), although a direct comparison with IFN-alpha only treated patients needs to be undertaken in larger numbers.

Ribavirin (virazole) is an orally administered nucleoside analogue which acts by inhibiting the synthesis of DNA. In a pilot study lasting six months, moderate reductions in ALT and HBV-DNA levels were observed (Fried et al 1994). Thymosin, a hormone-like acidic polypeptide, produced by the epithelial cells in the thymus, is known to promote IFN-alpha, IFN-gamma and IL-2 receptor expression (Goldstein et al 1972, Marshall et al 1981, Mutchnick et al 1982). Necrosis, liver inflammation and hepatocyte damage have been reduced in a small number of patients treated with thymosin alpha-1 and thymosin fraction 5, as well as indications of reduced viral replication (Mutchnick et al 1991). Both thymosin and ribavirin have been associated with mild adverse effects. The potential for mitochondrial damage by many of the nucleoside analogues deters extensive testing (Zoulim and Trepo 1994). Lamivudine however, is a drug that does not pass the mitochodrial membrane (Chang et al 1992) and has shown promising results in early clinical trials on HIV and HIV/HBV (co)infected patients. It is well tolerated for 28 days in coinfected patients administered 600mg once daily who showed a drastic drop in HBV DNA (as measured by the Abbott HBV-DNA kit) after 1 day (Schalm et al 1995). The long term safety and efficacy of these treatments is yet to be determined.

Phyllanthus niruri, a plant extract, has been found to inhibit DNA polymerase and bind WHsAg in vitro but also to clear WHsAg in experimentally infected animals and cause a drop in titre in chronically infected animals when injected intraperitoneally (Venkateswaran et al 1987). Fialuridine (FIAU) has been used on woodchucks to test the toxicity of the drug. It was found to cause defective mitochondrial DNA replication resulting in altered energy metabolism and eventually organ failure (Lewis et al 1997). To date, few drugs have undergone preclinical trials in the woodchuck due to difficulties in assessing their effects on the cellular and humoral immune response

but progress in this area would help screen candidate drugs for use in the treatment of hepatitis B infection .

Aim of the Thesis

The WHV infected woodchuck is a good animal model in which to study the pattern of hepadnaviral infection because viral antigen expression most closely resembles HBV and therefore it is thought that pathogenesis and disease outcome are achieved via the same mechanisms. Due to these similarities during the course of infection, it is thought that the immune response acts as the intercessor between the manifestations of liver injury and the virus, rather than the latter achieving its cytopathic effects directly (Cote et al 1991, Cote et al 1992). Although the woodchuck is employed as a suitable model for antiviral drug testing and immunoassay development, its potential for studying the immune response in viral infections, particularly hepatitis B, has not been exploited partly due to its reputation for ferocity during handling. As a result no previous work had been published in this area when work first began.

This study aims to explore the scope of this undefined area by designing assays to assess the cellular immune response and to find ways to manipulate it in order to temper its strength or augment it. Firstly, the conditions necessary to culture woodchuck lymphocytes were determined so that proliferation assays could be carried out as a means of assessing the effectiveness of different treatments on T cell activity. Due to the important role thought to be played by HBcAg in HBV infection work centred on its counterpart in the woodchuck, WHcAg. The T cell response to WHcAg was investigated using 23 overlapping peptides to identify an immunodominant epitope in chronically infected animals.

Different approaches to stimulating the immune response were under trial. The treatments tested acted at different stages of the immune response. Tucaresol, an immunopotentiating drug, was used to increase the interaction between APCs and Th cells and therefore targeted the second stage of the response. The aim of this study was to eradicate WHV DNA from the serum; GGT was used to monitor the development of HCC and the effect on the immune response was assessed using proliferative T cell responses.

The same parameters as in the tucaresol experiments were used to assess the effect of IL-12, a cytokine known to favour the Th1 subset of CD4 cells and in thus doing enhancing CD8 function, which acts at the third stage of the immune response.

The adoptive transfer of peptide specific T cells was attempted in order to boost the overall T cell response.

All the putative treatments represent novel approaches to HBV therapy as a result of our growing understanding of the immunopathogenesis of hepadnaviral infections.

Chapter 2

The Determination of Assay Conditions to Assess Woodchuck T Lymphocyte Responses

Published :

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(1997) Journal of Medical Virology 52:128-135

2.1 Introduction

In order to study the involvement of the immune system of the woodchucks during WHV infection, it is essential to be able to assess the cellular immune response to both mitogens and WHV specific antigens. An established protocol exists for human (Boyum 1968), mouse and other animal lymphocytes which involves isolation of the peripheral blood lymphocytes (PBL) on separation media and their growth in specific culture conditions. No such system exists for the woodchuck PBLs primarily because this aspect of the disease model has not been addressed. Some work has been done on the woodchuck system using conditions established for human PBLs but levels of radioactive label incorporation were very low in comparison to other animal systems (Korba *et al* 1988). In this section of the study each stage will be re-examined and the optimal conditions determined. This system can then be used throughout the course of infection or during a clinical trial of drugs, which can manipulate the immune response, to assess changes in PBL responsiveness.

2.2 Materials

Woodchucks: adult woodchucks were trapped in New York State, USA (North Eastern Wildlife, South Plymouth, New York, USA) and maintained in steel cages in our housing facility. They were fed rabbit pellets (Special Diet Services), fresh vegetables and water ad libitum. All animals used in these experiments are summarised in table 4. Animals were divided into two groups : chronically infected (C) and

ANIMAL NUMBER	WHV DNA STATUS (POSITIVE +/NEGATIVE -)	CHRONICALLY INFECTED (C),UNINFECTED (U)
W30	-	U
W31	-	U
W32	-	U
W34	-	U
W938	-	U
W939	-	U
W859	-	U
W867	-	U
W876	-	U
W406	-	U
W407	-	U
W520	-	U
W524	-	U
W26	+	C
W360	+	C
W363	+	C
W405	+	C
W414	+	C
W507	+	C
W518	+	C
W526	+	C
W527	+	C
W540	+	C
W556	+	C
W933	+	C
W935	+	C
W936	+	C
W937	+	C

Table 4. WHV DNA Status of All Animals Used in Cell Proliferation Assays (C-chronically infected woodchuck, U- uninfected woodchuck)

uninfected (U). **Uninfected animals were defined as being WHV DNA negative, WHeAg negative and anti-WHc negative. Chronically infected animals were defined as being WHV DNA positive, WHeAg positve and anti-WHc positive.**

Animals were anaesthetised by intramuscular injection of ketamine hydrochloride (25mg/kg) (Parke-Davis Medical) and xylazine (5mg/kg) (Southern Veterinary Supplies) and blood samples obtained by venepuncture of the hind legs.

Separation Media : Ficoll Paque (Pharmacia), Percoll (Pharmacia) and analytical grade Metrizamide (Nycomed) were used.

Whole blood cell counts were carried out on a Bayer Technicon H*2 machine.

Medium : RPMI medium (Imperial Labs) and Clicks Extra High Amino Acid medium (Irvine Scientific) (Appendix I) were used.

Supplement : Foetal calf serum (JRH Biosciences, Gibco, BRL, Sigma), rat serum (ICN), mouse serum (ICN), rabbit serum (ICN) and sheep serum (ICN) were used.

Mitogens : Phytohaemmagglutinin (PHA)(Sigma), concanavalin A (Con. A) (Sigma) and lipopolysaccharide (LPS)(Sigma)were used.

Radioactive Label: [^{3}H] Thymidine (Amersham International), [^{3}H] uridine (Amersham International) and [^{3}H] bromodeoxyuridine (Amersham International) were used.

2-Mercaptoethanol (2-ME)(Sigma), glass fibre filters (Packard), dimethyl sulphoxide (DMSO)(Sigma), Matrix 96 computer (Packard), cell culture plates (Corning), red blood cell lysing buffer (Sigma), heparin (Leo Laboratories), 1000U/ml stock solution vacuum bag sealer (PIFCO), cryovials (Corning) and a Packard Filtermate 196 harvester were used .

2.3 Methods

Optimal conditions for measuring T cell proliferation were obtained by testing each of the following conditions.

Section 2.3.1 Separation of Peripheral Blood Lymphocytes from Whole Blood

The woodchuck was bled from the antecubital vein; between 2 and 5 mls of blood were extracted and 10U/ml heparin added. The blood was placed on separation media (either Percoll, Ficoll-Paque or metrizamide) at a dilution of 1 in 2, 1 in 3 and undiluted. After centrifugation at 1000rpm for 30' the cells were removed, washed and counted. Percentage recovery was calculated using the formula : number of cells present after separation/number of cells present before separation x 100. The recovery percentages were compared for the different media. Cell viability was checked using trypan blue and all assays were conducted in triplicate. Changes in percentage composition of whole blood were determined using blood samples from 3 animals which were analysed using a Bayer Technicon H*2 machine.

Section 2.3.2. Woodchuck PBLs Cultured Under Conditions Normally Used for Human Lymphocytes

After separation cells were washed three times at 1800, 1500 and 1200rpm in RPMI 1640 medium and resuspended at 1 x 10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS. 1 x 10^5 cells (100ul) were plated out in a round bottomed 96-well plate to which PHA, Con A or LPS was added at three concentrations - 1, 5 and 10ug/ml. Cultures were incubated at 37°C with 5% CO_2 in a sealed bag for 3 days and pulsed with ^{3}H-thymidine (Tdr) for 18 hours. Cells were harvested onto glass fibre filters and allowed to dry before measurement on a Matrix 96 computer.

Section 2.3.3. The Effect of Substituting Uridine (Udr) as Radiolabel on the Proliferation of Woodchuck PBLs

The same conditions were used as in section 2.3.2 to culture the lymphocytes but Udr was used to pulse the cells for 6 hours.

Section 2.3.4.The Effect of Substituting Different Serum Supplements on the Proliferation of Woodchuck PBLs

Conditions used were the same as in section 2.3.2 but serum from other sources was also used at 10% concentration with RPMI medium, in particular rat, rabbit, mouse and sheep.

Section 2.3.5.The Effect of Substituting Clicks Medium as Culture Medium on the Proliferation of Woodchuck PBLs

Clicks medium is a highly enriched medium and therefore only 0.5% serum supplement is required. Mouse, rat, sheep and rabbit serum were tested using this medium.

Section 2.3.6.The Effect of Different Percentages of Serum Supplement on the Proliferation of Woodchuck PBLs

WCS, from an uninfected animal (W867) and mouse serum were used to supplement Clicks medium at two different concentrations- 0.5% and 10%.

Section 2.3.7.The Effect of Addition of 2-Mercaptoethanol (2-ME) to the Culture Medium on the Proliferation of Woodchuck PBLs

A stock solution of 5 x 10^{-5} M 2-ME was prepared in RPMI and filter sterilised. The final concentration of 2-ME in the medium was $2x10^{-5}$ M.

Section 2.3.8.The Effect of Different Mitogens on the Proliferation of Woodchuck PBLs

Different mitogens were used to stimulate woodchuck PBLs namely PHA, Con A and LPS. Based on preliminary data this was restricted to a comparison between PHA and

Con A. The mitogens were tested at concentrations of 0.25-20ug/ml. Each mitogen was made up in RPMI medium and at a stock solution of 1mg/ml and then diluted to the relevant concentration.

Section 2.3.9.The Effect of Increasing the Incubation Time on the Proliferation of Woodchuck PBLs

PBLs were cultured for a 4 and 7 day period and their incorporation of Udr compared.

Section 2.3.10.The Effect of Substituting Different Radiolabels in the Culture Medium on the Proliferation of Woodchuck PBLs

^{3}H-thymidine incorporation was tested initially to measure T cell proliferation as it is used routinely for mouse and human lymphocytes. In addition the incorporation of ^{3}H-uridine (specific activity 27Ci/mmol) and ^{3}H-5-bromo-2'-deoxyuridine (specific activity 27 Ci/mmol) was also tested.

Section 2.3.11.Optimal Conditions for Culturing Woodchuck PBLs

To confirm the optimal culture conditions for woodchuck lymphocytes 10 chronically infected and 10 uninfected animals were bled in order to obtain a blood sample. Lymphocytes were separated using Ficoll-Paque after dilution 1 in 3 in RPMI 1640 medium. Cells were washed three times, resuspended in Clicks medium supplemented with 0.5% WCS and 2-ME, plated out and stimulated with PHA. After 4 days of

incubation at 37°C with 5% CO_2, cells were pulsed with Udr for the last 6 hours of culture and then harvested.

Presentation of the Data

Incorporation of radioactive label is measured as counts per minute (cpm). Each assay was conducted in triplicate and the mean cpm calculated. The mean cpm from 5 animals was then calculated along with the standard error of the mean (S.E.M.). This raw data is presented in Appendix II. The stimulation index (S.I.) was also calculated for each set of cpm data using the following formula:

$$\frac{\text{mean cpm obtained from PBLs in the presence of mitogen}}{\text{mean cpm obtained from PBLs in the absence of mitogen}}$$

The mean S.I. for 5 animals was calculated along with the S.E.M. and this data is presented in a graphical format in the results section.

Statistical Analysis

The Mann-Whitney U-test was performed using the Minitab software to compare data from chronically infected and uninfected animals for each set of conditions. A p value was obtained to indicate the level of significance. p values less than 0.05 were considered to be significant. In addition the Kruskall-Wallis test was performed in order to compare 3 or more groups of data and values less than 0.05 were considered to be significant.

2.4 Results

Section 2.4.1 Separation of PBL from Whole Blood

The recovery of lymphocytes using different commercially available separation media was compared. Separation of 2mls of blood diluted 1 in 3, on Ficoll-Paque yielded 16.5 x 10^6 lymphocytes whereas Percoll and metrizamide yielded 3.7 x 10^6 and 0.15 x 10^6 cells respectively. The total peripheral blood mononuclear cells/ml blood was 8.65 x 10^6. The percentage recovery of peripheral blood mononuclear cells from separation on Ficoll-Paque was 95%. Cell viability was determined as 90%.

WBC	% Before Separation					% After Separation				
	1	2	3	$\bar{x}$	S.E.M.	1	2	3	$\bar{x}$	S.E.M
Neutrophil	16.7	50.2	47.1	38	18.5	14.4	35.5	33.8	27.9	11.7
Lymphocyte	61.7	33.4	29.9	41.7	17.4	82.3	51.3	60.8	64.8	15.8
Monocyte	9.7	11.9	14.6	12	2.4	2.1	5.7	4.9	4.2	1.8
Eosinophil	0.1	0.7	0.3	0.4	0.3	0.1	0.7	0.3	0.4	0.3
Basophil	0.4	0.6	0.2	0.4	0.2	0.4	0.6	0.1	0.4	0.3
Leucocyte	11.4	3.2	8	7.5	4.1	0.7	0.5	0.2	0.5	0.2

Table 5. Change in the percentage composition of white blood cells (WBC) after Ficoll-Paque separation. Monocytes are the significant loss. Results are the mean ($\bar{x}$) of triplicate values obtained from 3 chronically infected woodchucks with the S.E.M.

2.4.2 Woodchuck PBLs Cultured Under Conditions Normally Used for Human Lymphocytes

The first set of experiments carried out employed RPMI medium with 10% FCS as supplement for a 3 day incubation using thymidine (Tdr) to pulse the cells for 18 hours. These conditions were based on what was found to be successful for human and mouse cells. Table 6 compares the background incorporation of label in PBLs from chronically infected and uninfected woodchucks under these conditions.

Woodchuck (Number)	Basal Incorporation (Mean cpm ± S.E.M.)		
	Exp. 1	Exp. 2	Exp. 3
Chronically Infected (5)	84 ± 48	507 ± 644	507 ± 644
Uninfected (5)	666 ± 517	695 ± 794	695 ± 794
p Value	0.04	0.08	0.08

Table 6. Basal level of incorporation of Tdr into unstimulated woodchuck PBLs from chronically infected and uninfected animals incubated in RPMI medium supplemented with 10% FCS. Mann-Whitney U-test p values <0.05 were considered significant.

Under these conditions Con A at 1ug/ml and LPS at 5 and 10ug/ml produced a significant difference in S.I. values between chronically infected and uninfected animals (figure 9). However, when the three mitogens were compared (figures 10 and 11) a significantly different S.I. was observed in chronically infected animals only. (Mean cpm data in Appendix II, tables 1-3)

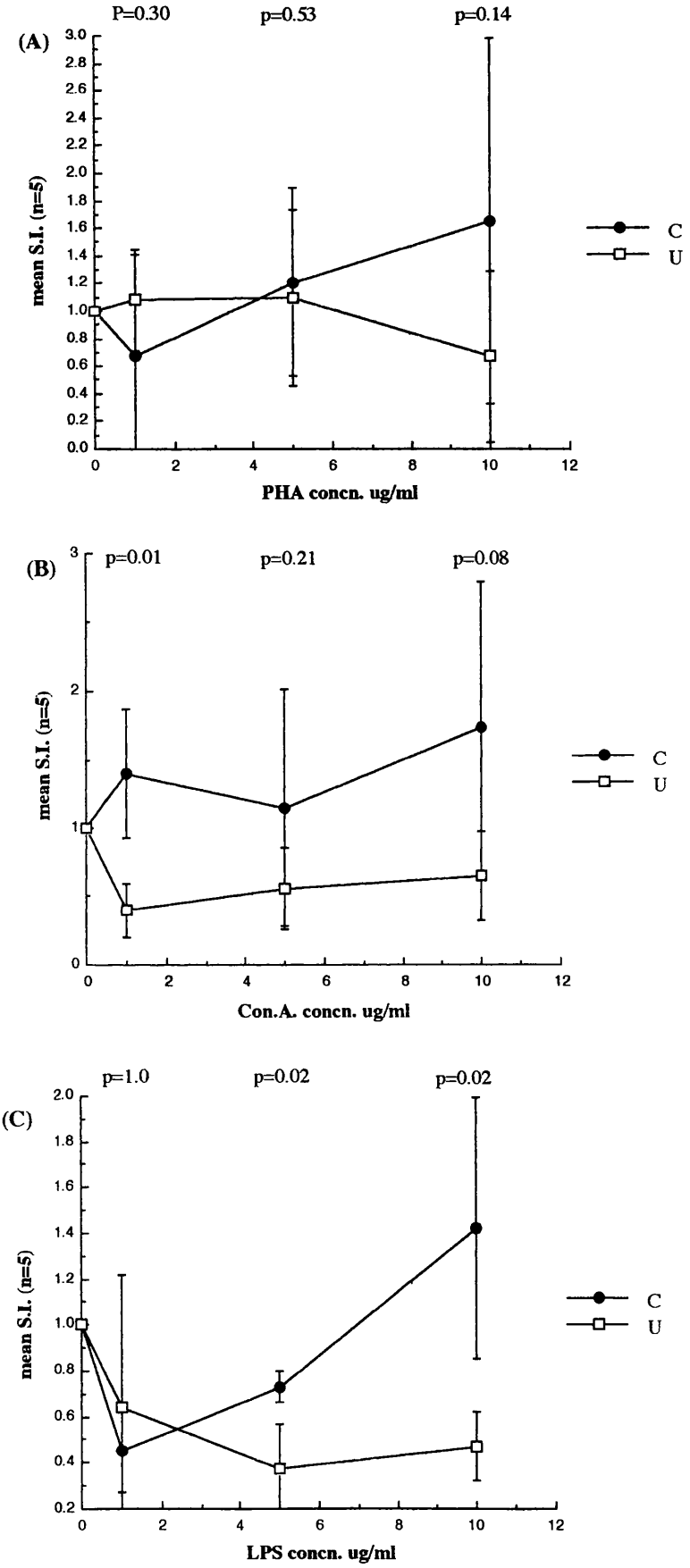


Figure 9. Graphical representation of the data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to A) PHA, B) Con A and C) LPS. PBLs were incubated in RPMI 1640 medium with 10% FCS for 3 days. p Values < 0.05 were considered significant.

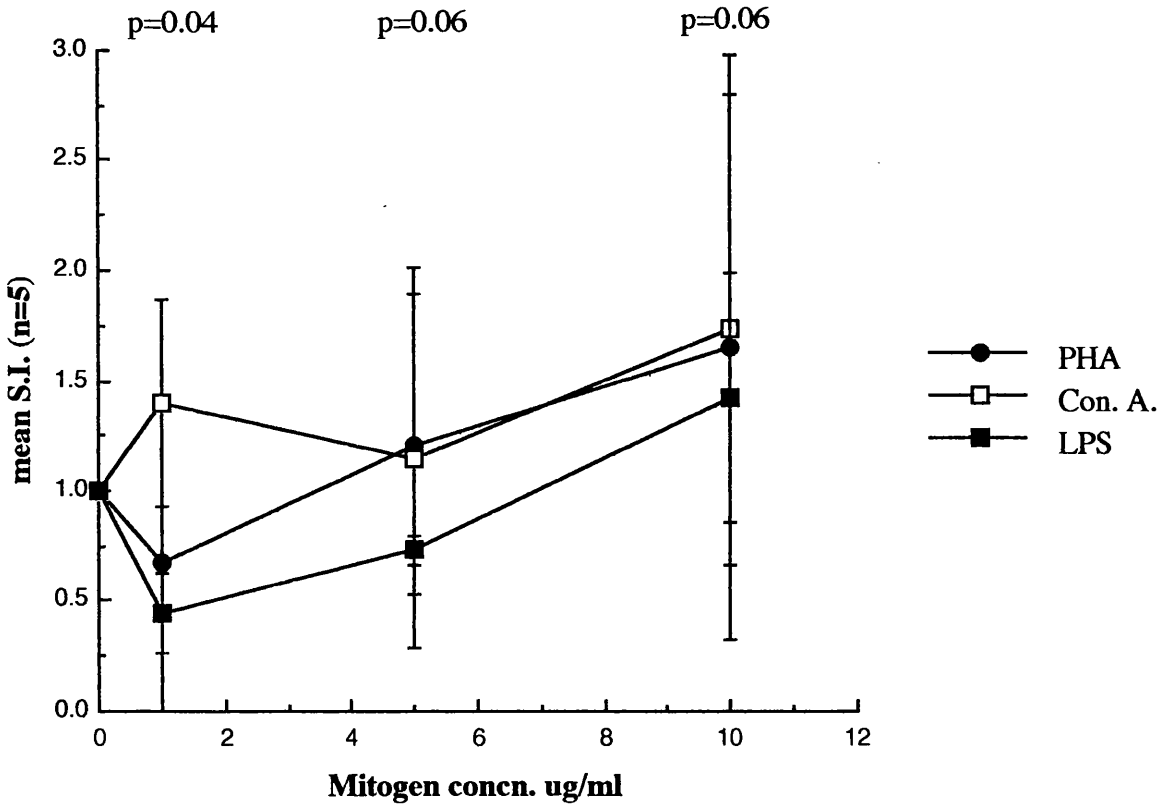


Figure 10. Graphical representation of data showing the different responses of PBLs from chronically infected woodchucks to 3 different mitogens. PBLs were incubated in RPM1 1640 medium with 10% FCS for 3 days. p values <0.05 were considered significant.

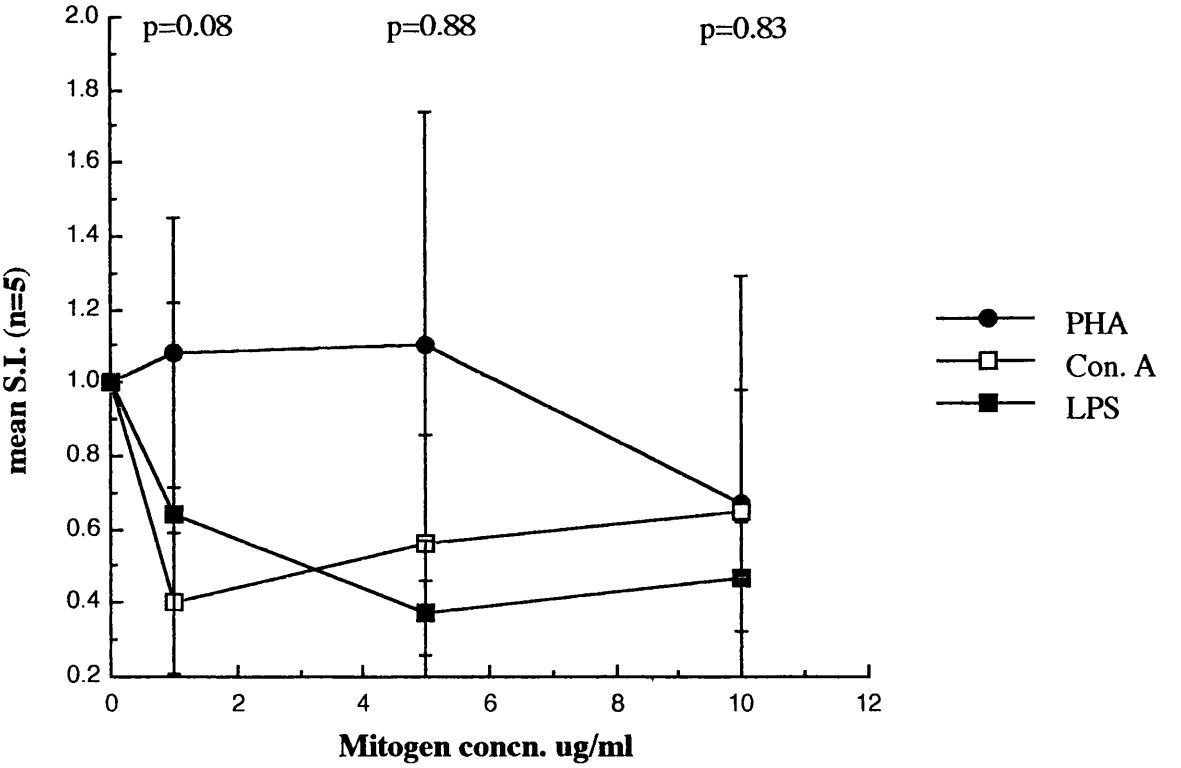


Figure 11. Graphical representation of data showing the different responses of PBLs from uninfected woodchucks to 3 different mitogens. PBLs were incubated in RPM1 1640 medium with 10% FCS for 3 days. p values <0.05 were considered significant.

2.4.3. The Effect of Using Uridine as Radiolabel on the Proliferation of Woodchuck PBLs

The same conditions were used in a series of experiments employing uridine (Udr) as the radiolabel. Table 7 summarises the background level of incorporation in chronically infected and uninfected animals.

Woodchuck (Number)	Basal Incorporation (Mean cpm ± S.E.M.)		
	Exp. 1	Exp. 2	Exp. 3
Chronically Infected (5)	306 ± 189	199 ± 71	339 ± 256
Uninfected (5)	437 ± 370	553 ± 381	644 ± 352
p Value	1	0.53	0.21

Table 7. Basal level of incorporation of Udr into unstimulated woodchuck PBLs from chronically infected and uninfected animals incubated in RPMI medium supplemented with 10% FCS. Mann-Whitney U-test p values <0.05 were considered significant.

Under these conditions Con A at 1 and 5ug/ml produced a significant difference in S.I. values between chronically infected and uninfected animals (figure 12). However, when the three mitogens were compared in chronically infected and uninfected animals (figures 13 and 14) significant differences were observed in chronically infected animals at 1 and 10ug/ml and at 5ug/ml in uninfected animals. (Mean cpm data in Appendix II, tables 4-6)

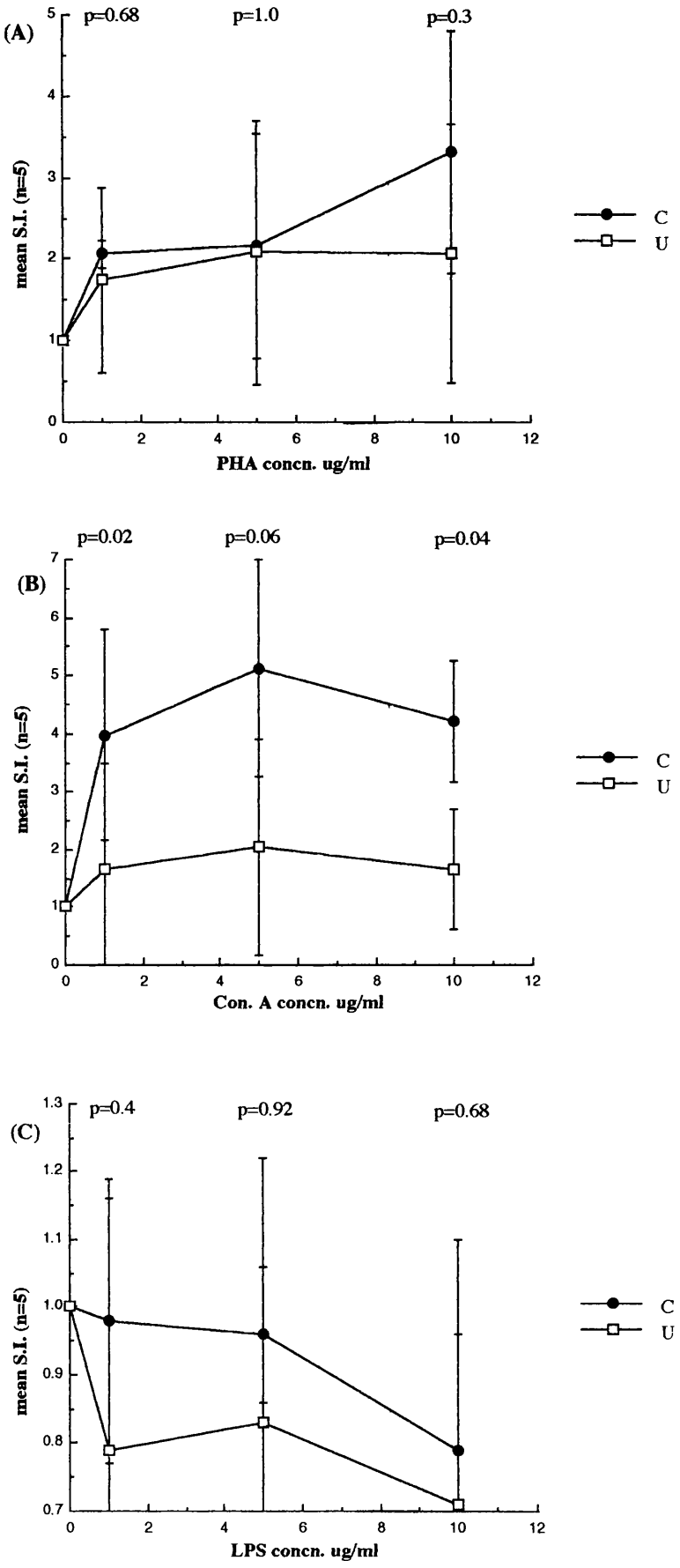


Figure 12. Graphical representation of the data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to A) PHA, B) Con A and C) LPS. PBLs were incubated in RPMI 1640 medium with 10% FCS and pulsed with Udr. p Values < 0.05 were considered significant.

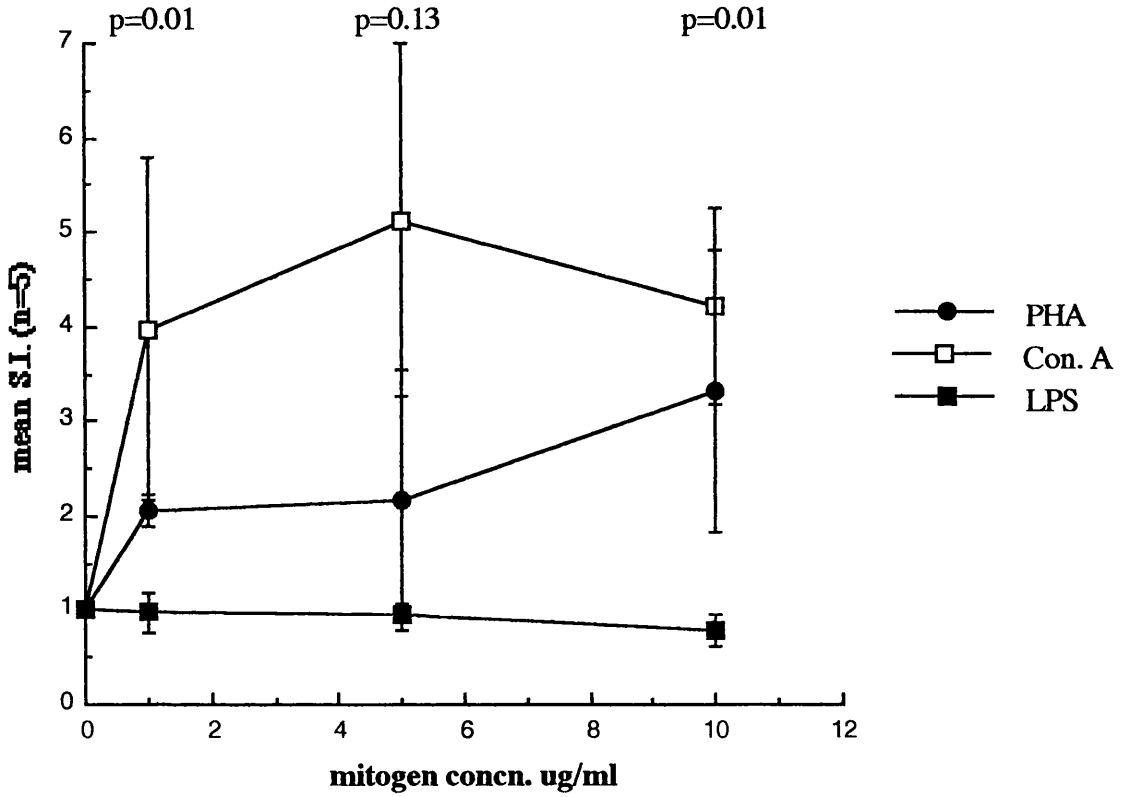


Figure 13. Graphical representation of data comparing the different responses of PBLs from chronically infected woodchucks to 3 different mitogens. PBLs were incubated in RPM1 1640 medium with 10% FCS and pulsed with Udr. p values <0.05 were considered significant.

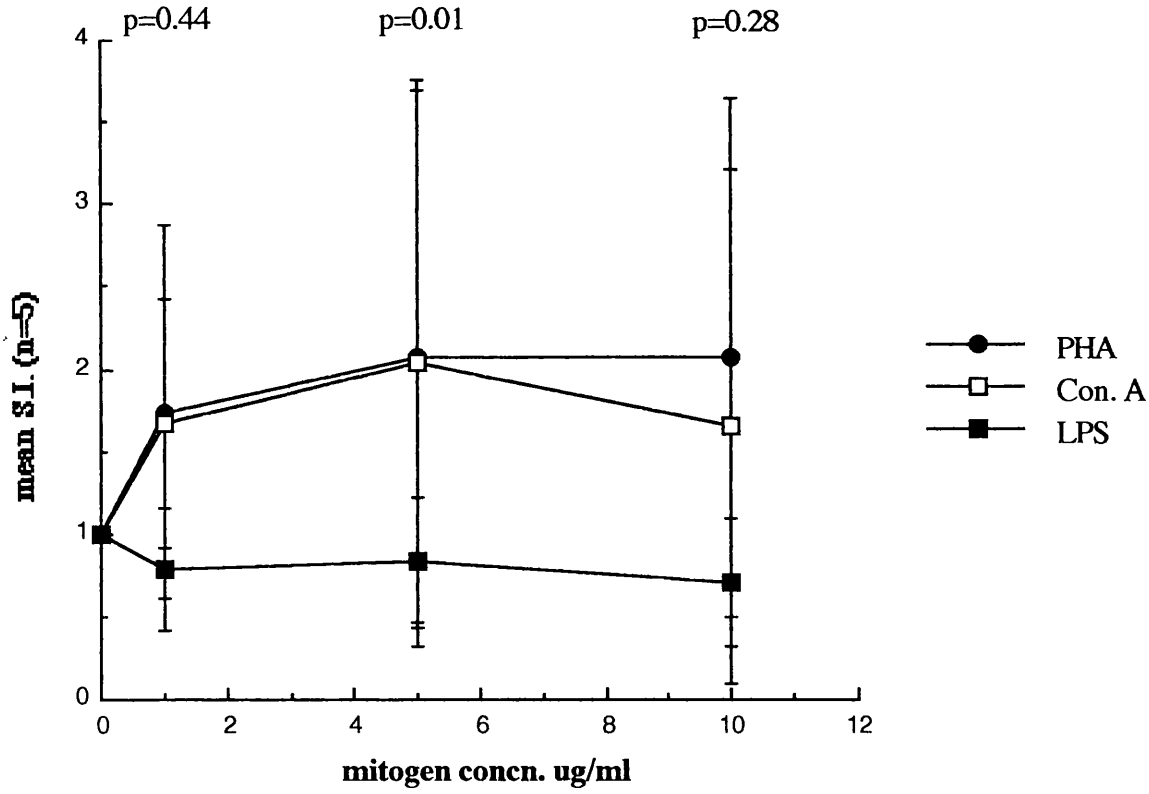


Figure 14. Graphical representation of data comparing the different responses of PBLs from uninfected woodchucks to 3 different mitogens. PBLs were incubated in RPM1 1640 medium with 10% FCS and pulsed with Udr. p values <0.05 were considered significant.

2.4.4. The Effect of Substituting Different Serum Supplements on the Proliferation of Woodchuck PBLs

Con A appeared to produce the best S.I. and was therefore used further to investigate the effect of different serum supplements on the original conditions. Sera from mouse, rat, rabbit and sheep was used in RPMI at 10%. Table 8 summarises the background level of incorporation of Tdr in PBLs from chronically infected and uninfected animals.

Woodchuck (Number)	Basal Incorporation (Mean cpm ± S.E.M.)			
	10% mouse	10% rat	10% sheep	10% rabbit
Chronically Infected (5)	183± 64	339 ± 127	906 ± 213	937 ± 414
Uninfected (5)	1015 ± 523	1015 ± 523	650 ± 638	576 ± 324
p Value	0.04	0.10	0.53	0.21

Table 8. Basal level of incorporation of Tdr into unstimulated woodchuck PBLs from chronically infected and uninfected animals incubated in RPMI medium supplemented with 10% serum from mouse, rat, sheep and rabbit. Mann-Whitney U-test p values <0.05 were considered significant.

A significant difference in background S.I.s was observed between chronically infected and uninfected animals using 10% mouse serum only (figure 15). A significant difference in S.I.s was observed in chronically infected animals using 1 and 10ug/ml Con A when all four serum supplements were compared (figures 16 and 17). In addition the S.I.s produced using mouse serum are higher than with the other supplements. (Mean cpm data in Appendix II, tables 7-10)

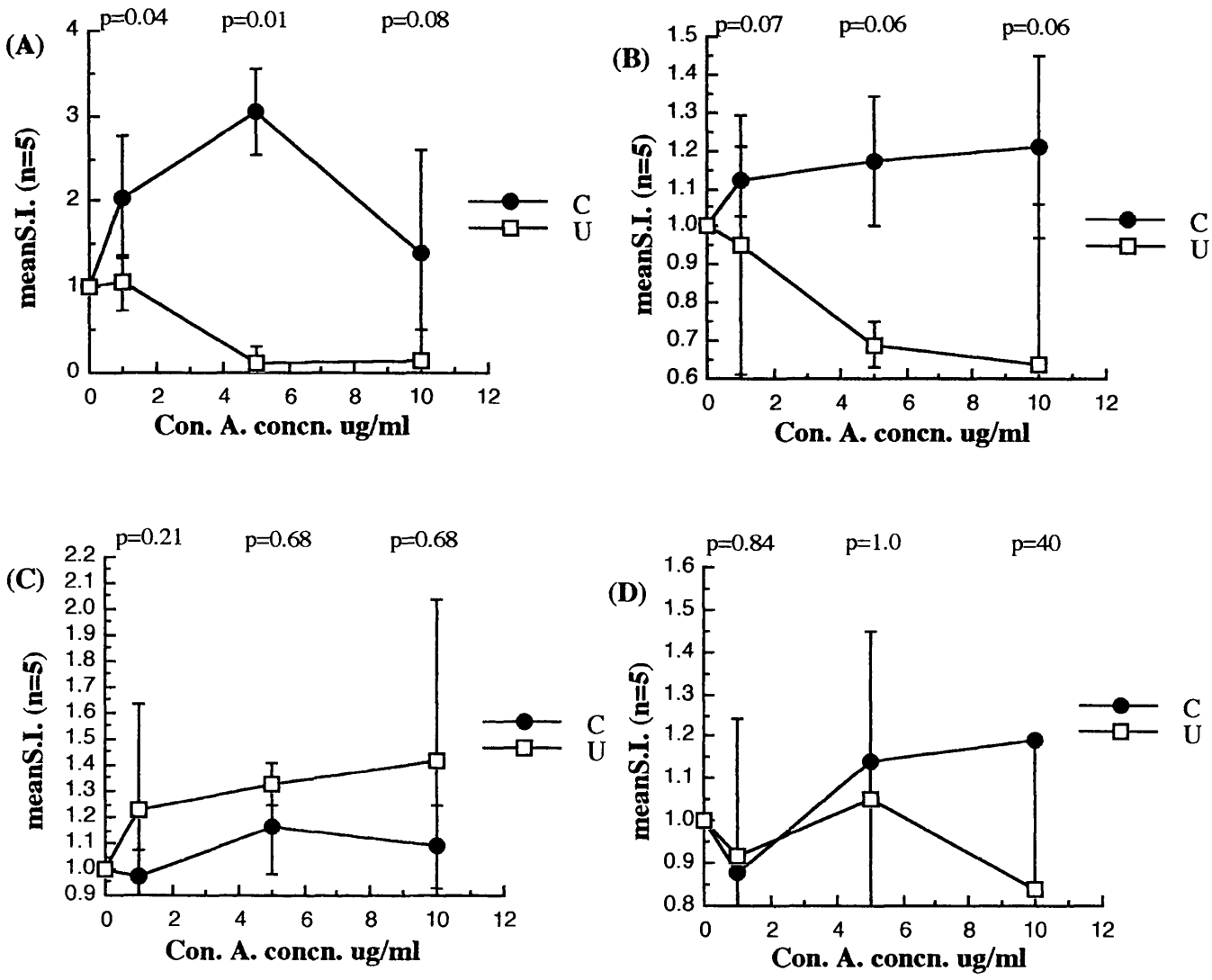


Figure 15. Graphical representation of data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to Con A. PBLs were incubated in RPMI 1640 medium with A) 10% mouse serum B) 10% rat serum C) 10% sheep serum and D) 10% rabbit serum and pulsed with Tdr. p values < 0.05 were considered significant.

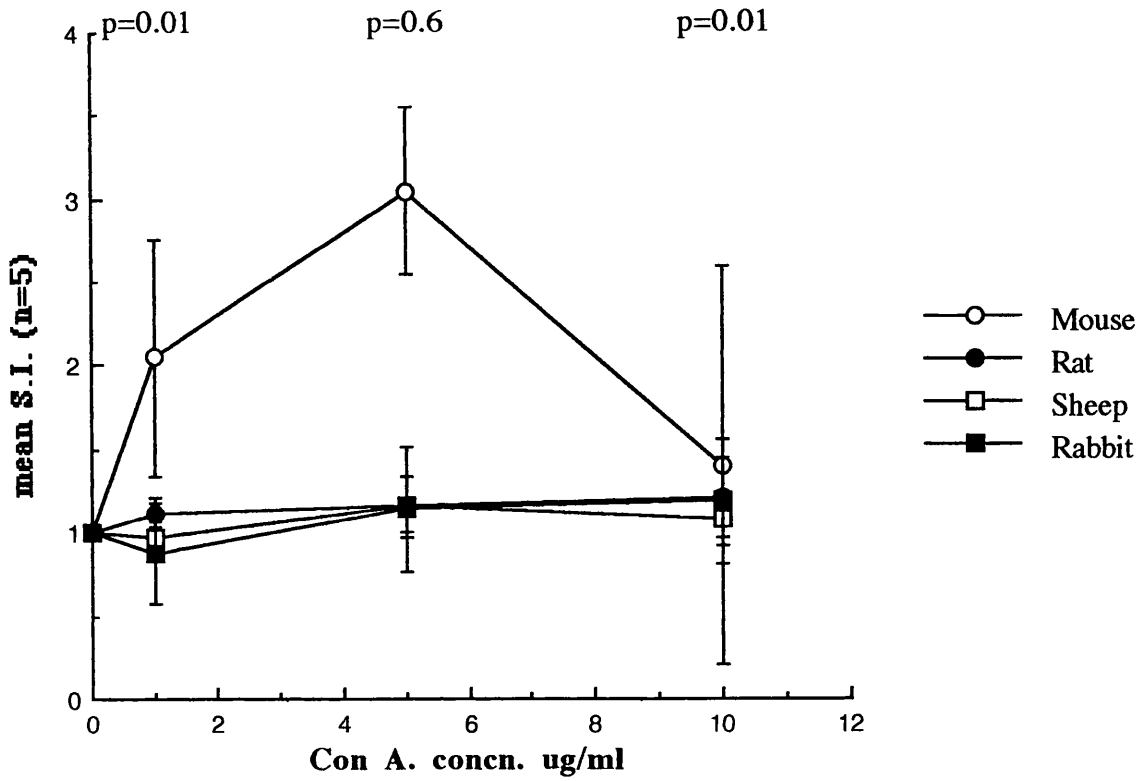


Figure 16. Graphical representation of the data comparing the different responses of PBLs from chronically infected woodchucks to Con A. PBLs were incubated in RPMI 1640 medium with 10% different serum supplements and pulsed with Tdr. p Values < 0.05 were considered significant.

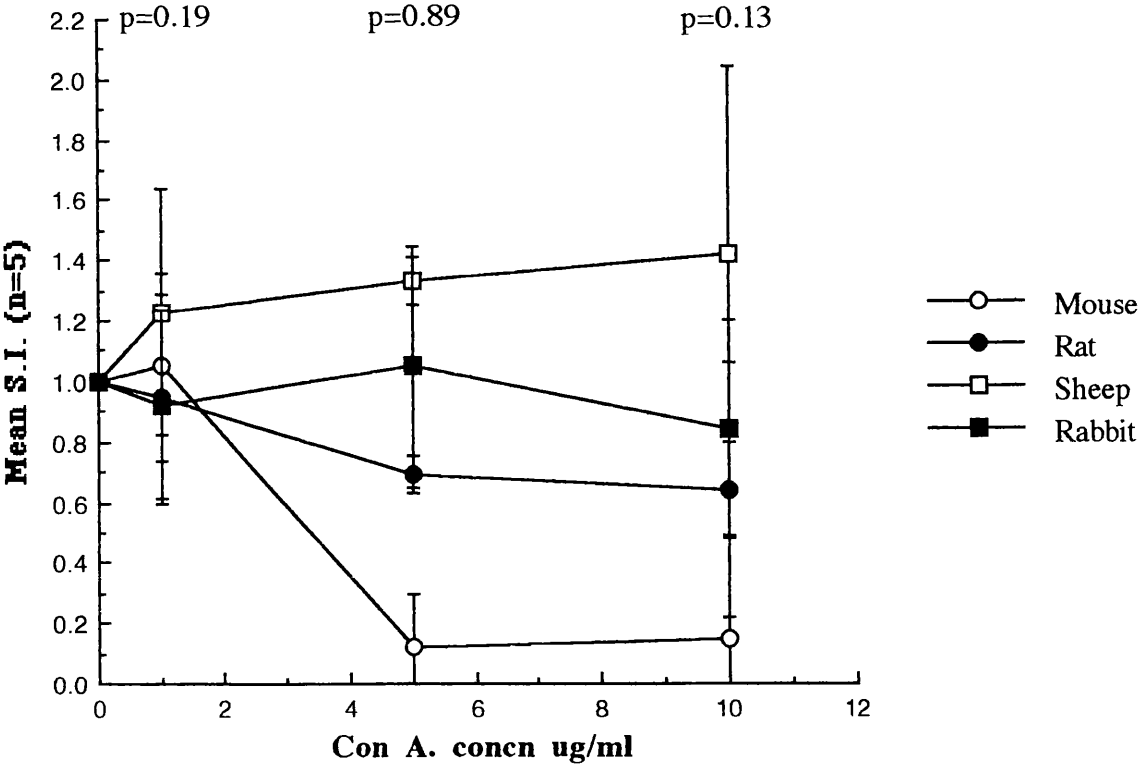


Figure 17. Graphical representation of the data comparing the different responses of PBLs from uninfected woodchucks to Con A. PBLs were incubated in RPMI 1640 medium with 10% different serum supplements and pulsed with Tdr. p Values < 0.05 were considered significant.

2.4.5. The Effect of Using Clicks medium as Culture medium on the Proliferation of Woodchuck PBLs

Clicks medium is highly enriched with amino acids (Appendix I) and normally serum is supplemented only at 0.5% in this medium and the cells incubated for a longer time period. Under these conditions the same four serum supplements were used at a percentage of 0.5% using Tdr to pulse the cells. Table 9 summarises the background level of incorporation of label into PBLs from both chronically infected and uninfected woodchucks.

Woodchuck (Number)	Basal Incorporation (Mean cpm ± S.E.M.)			
	0.5% mouse	0.5% rat	0.5% sheep	0.5% rabbit
Chronically Infected (5)	465 ± 345	954 ±.708	1489 ±1047	1111± 601
Uninfected (5)	1274 ± 290	1274 ± 290	1197 ± 317	1274 ± 290
p Value	0.01	0.40	0.83	1

Table 9. Basal level of incorporation of Tdr into unstimulated woodchuck PBLs from chronically infected and uninfected animals incubated in Clicks medium supplemented with 0.5% serum from mouse, rat, sheep and rabbit. Mann-Whitney U-test p values <0.05 were considered significant.

There was no significant difference in the S.I. values between chronically infected and uninfected animals using rat or sheep serum. A significant difference was observed between the two groups using mouse and rabbit serum with 1ug/ml Con A (figure 18). When all four sera were compared, using the Kruskall-Wallis test, there was a significant difference between them in both chronically infected and uninfected animals (figures 19 and 20). (Mean cpm data in Appendix II, tables 11-14)

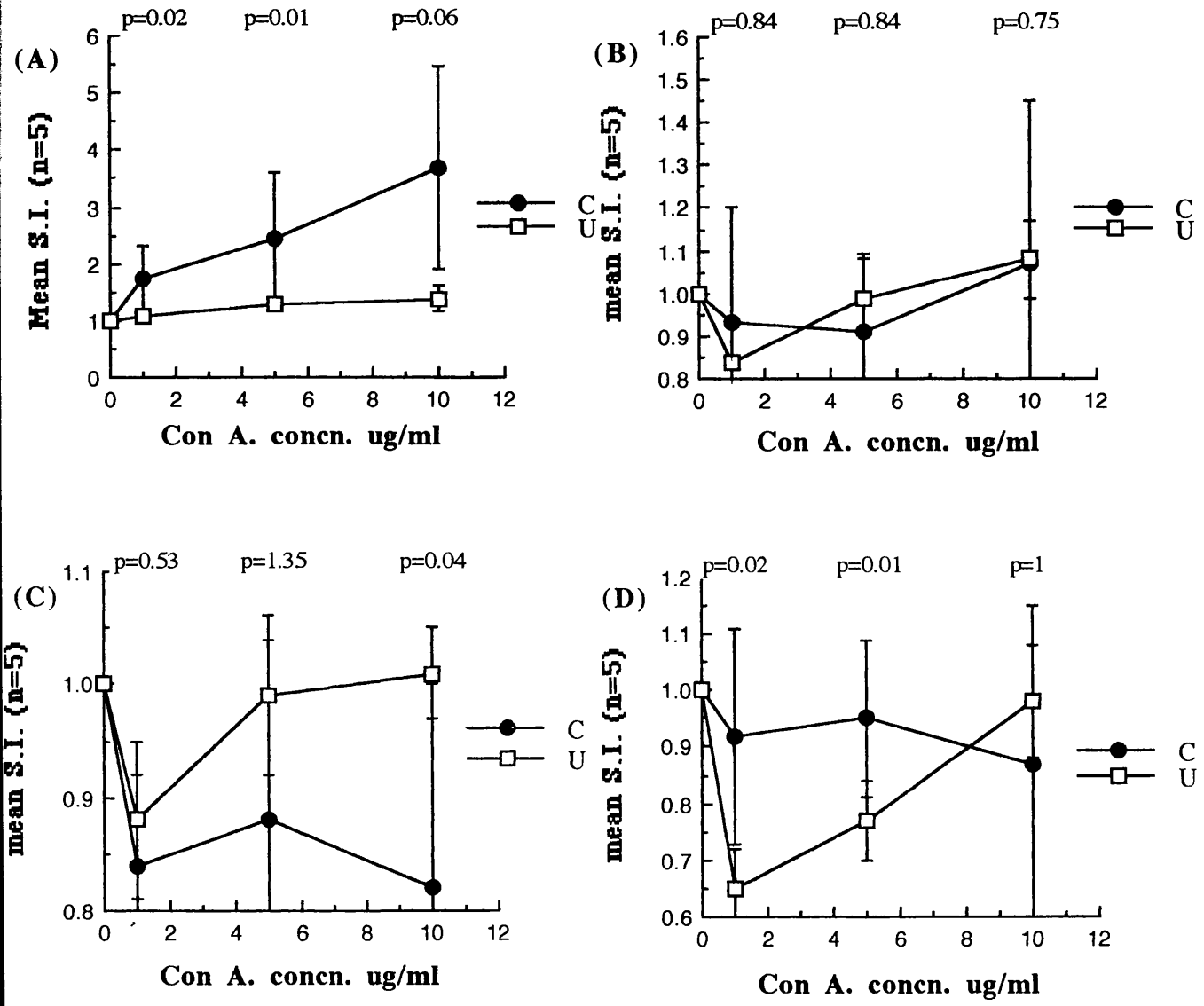


Figure 18. Graphical representation of the data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to Con A. PBLs were incubated in Clicks medium with A) 0.5% mouse serum B) 0.5% rat serum C) 0.5% sheep serum and D) 0.5% rabbit serum and pulsed with Tdr. p Values < 0.05 were considered significant.

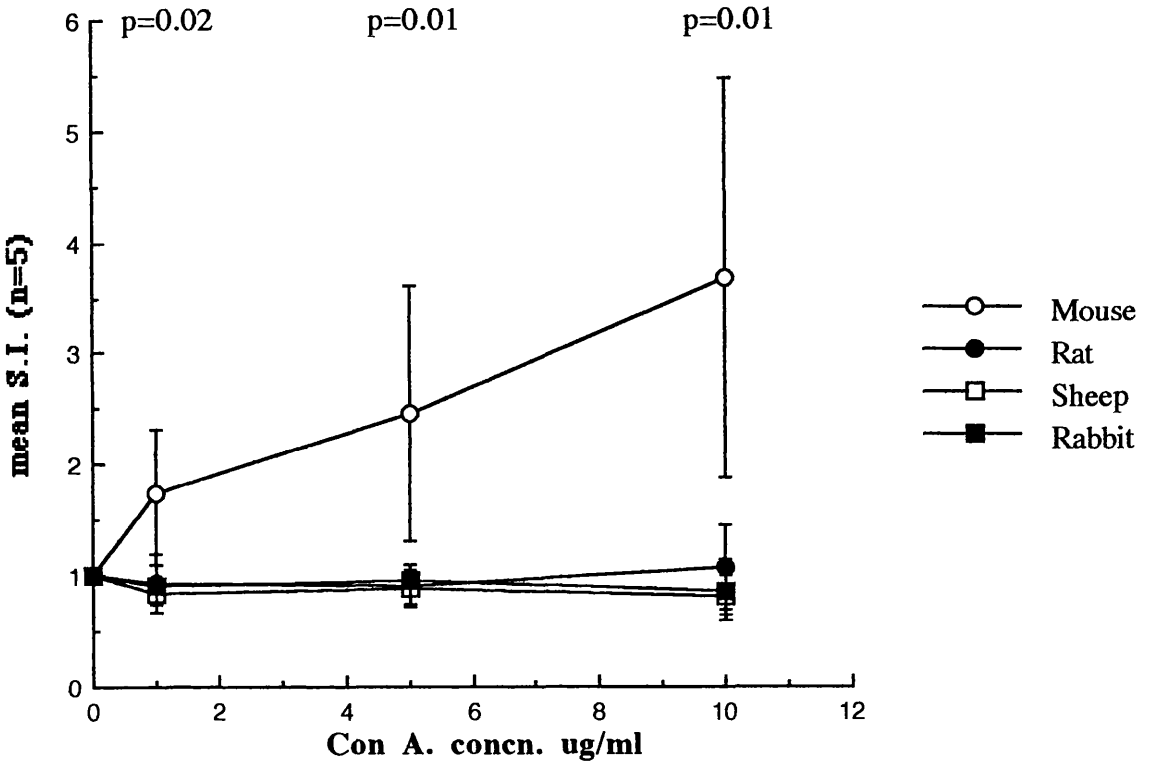


Figure 19. Graphical representation of the data comparing the different responses of PBLs from chronically infected woodchucks to Con A. PBLs were incubated in Clicks medium with 0.5% different serum supplements and pulsed with Tdr. p Values < 0.05 were considered significant.

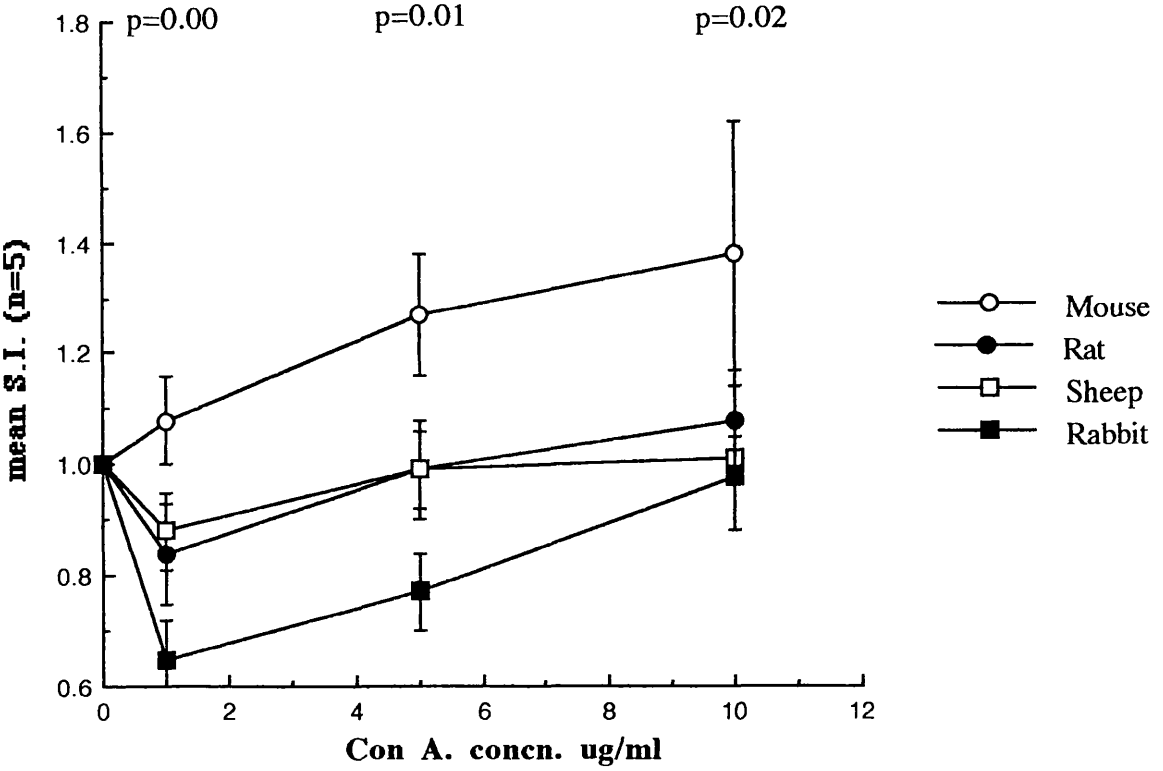


Figure 20. Graphical representation of the data comparing the different responses of PBLs from uninfected woodchucks to Con A. PBLs were incubated in Clicks medium with 0.5% different serum supplements and pulsed with Tdr. p Values < 0.05 were considered significant.

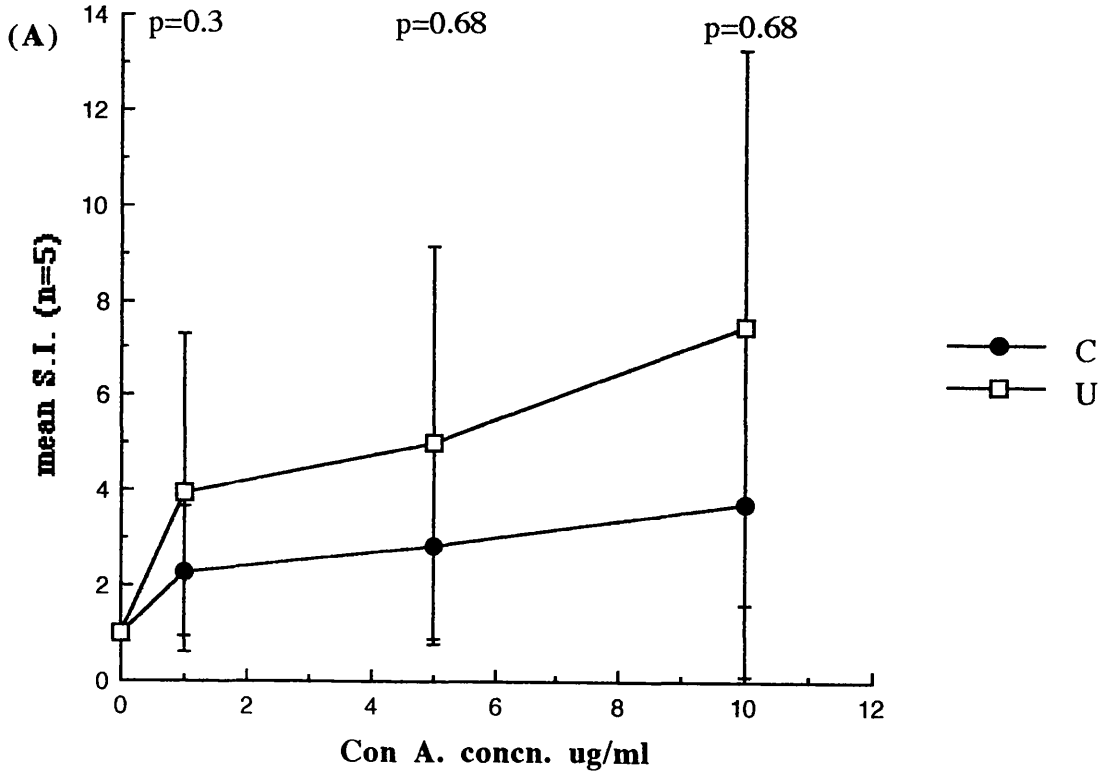
2.4.6. The Effect of Different Percentages of Serum Supplements on the Proliferation of Woodchuck PBLs

This medium was used to test woodchuck serum (WCS) and mouse serum (MS) at both 10% and 0.5% using Udr as the radiolabel. Table 10 summarises the background level of incorporation of Udr into PBLs from chronically infected and uninfected woodchucks.

Woodchuck (Number)	Basal Incorporation (Mean cpm ± S.E.M.)			
	10% MS		10% WCS	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Chronically Infected (5)	109 ± 110	99 ± 115	561 ± 199	865 ± 410
Uninfected (5)	61 ± 14	719 ± 176	141± 49	441 ± 392
p Value	0.01	0.83	0.09	0.01

Table 10. Basal level of incorporation of Udr into unstimulated woodchuck PBLs from chronically infected and uninfected animals incubated in Clicks medium supplemented with 10% serum from mouse and woodchuck. Mann-Whitney U-test p values <0.05 were considered significant.

There was no significant difference between chronically infected and uninfected animals using 10% MS with either Con A (figure 21) or PHA (figure 23) or using 10% WCS with ConA; a significant difference in S.I. values was observed with 5ug/ml PHA (figure 23). There was no significant difference between 10% WCS or 10% MS in chronically infected or uninfected animals using Con A (figure 22). However, using PHA there was a significant difference in the S.I. values obtained at 10ug/ml in chronically infected animals and at 5 and 10ug/ml in uninfected animals (figure 24). (Mean cpm data in Appendix II, tables 15-18)



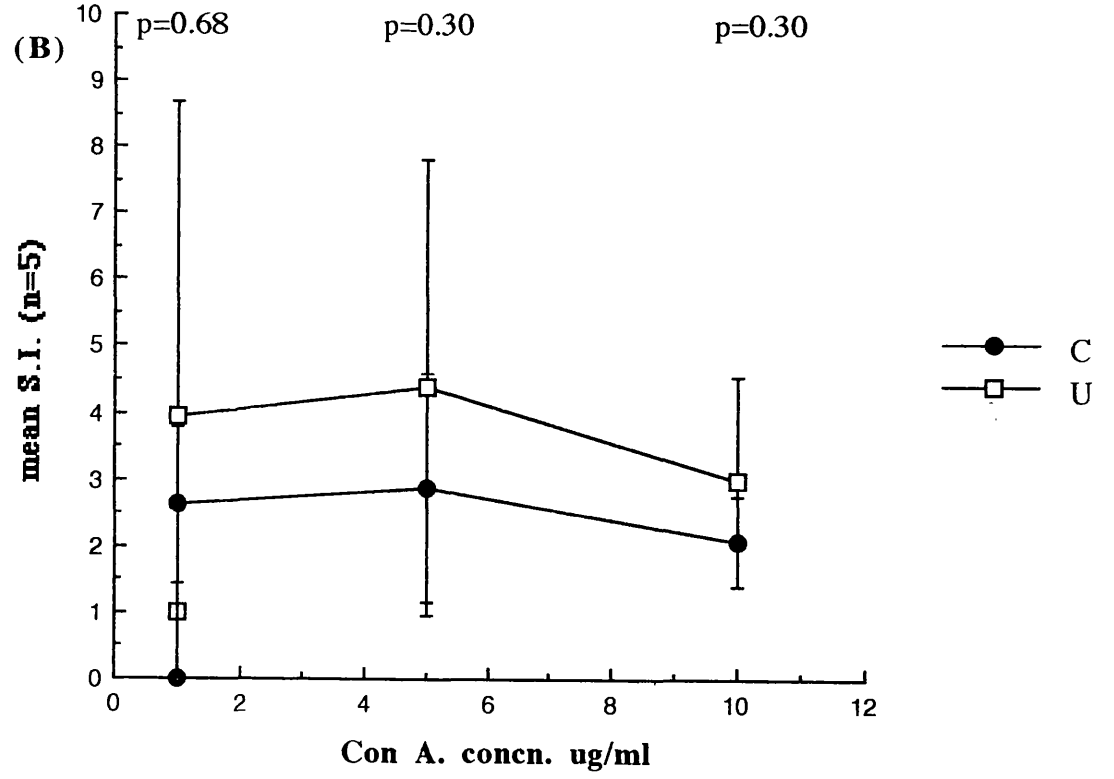
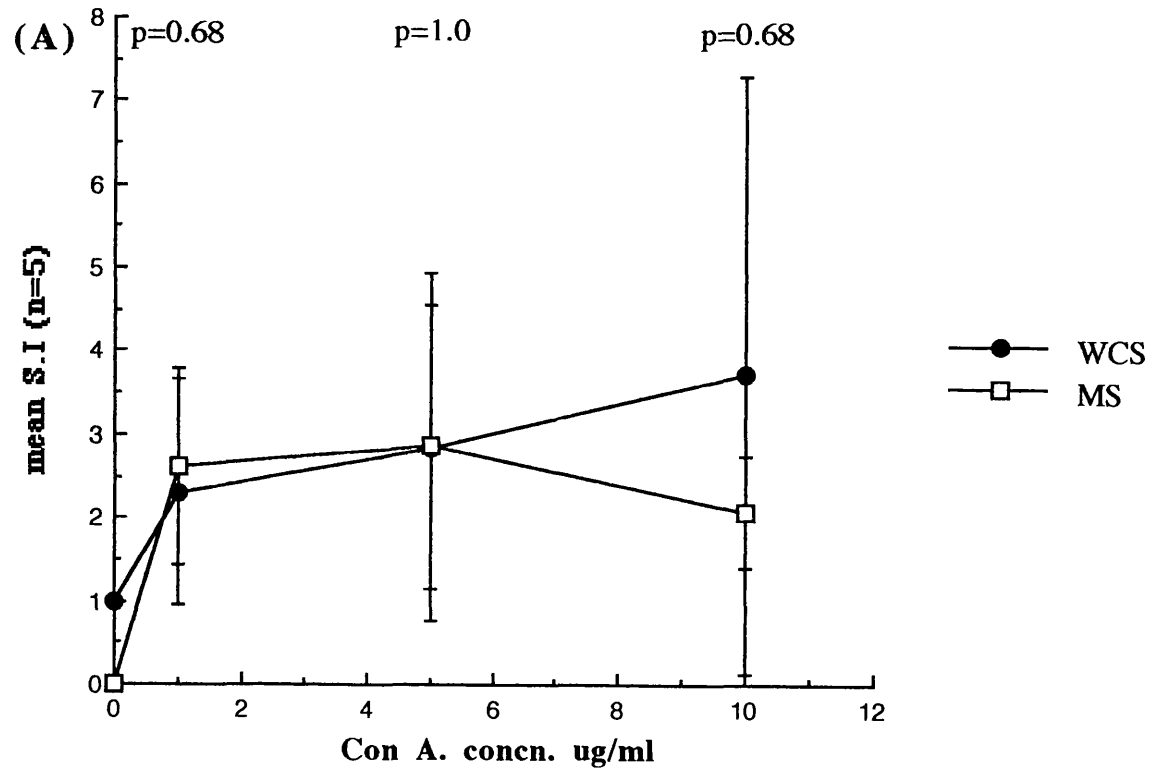


Figure 21. Graphical representation of the data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to Con A. PBLs were incubated in Clicks medium with A) 10% WCS and B) 10% MS and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.



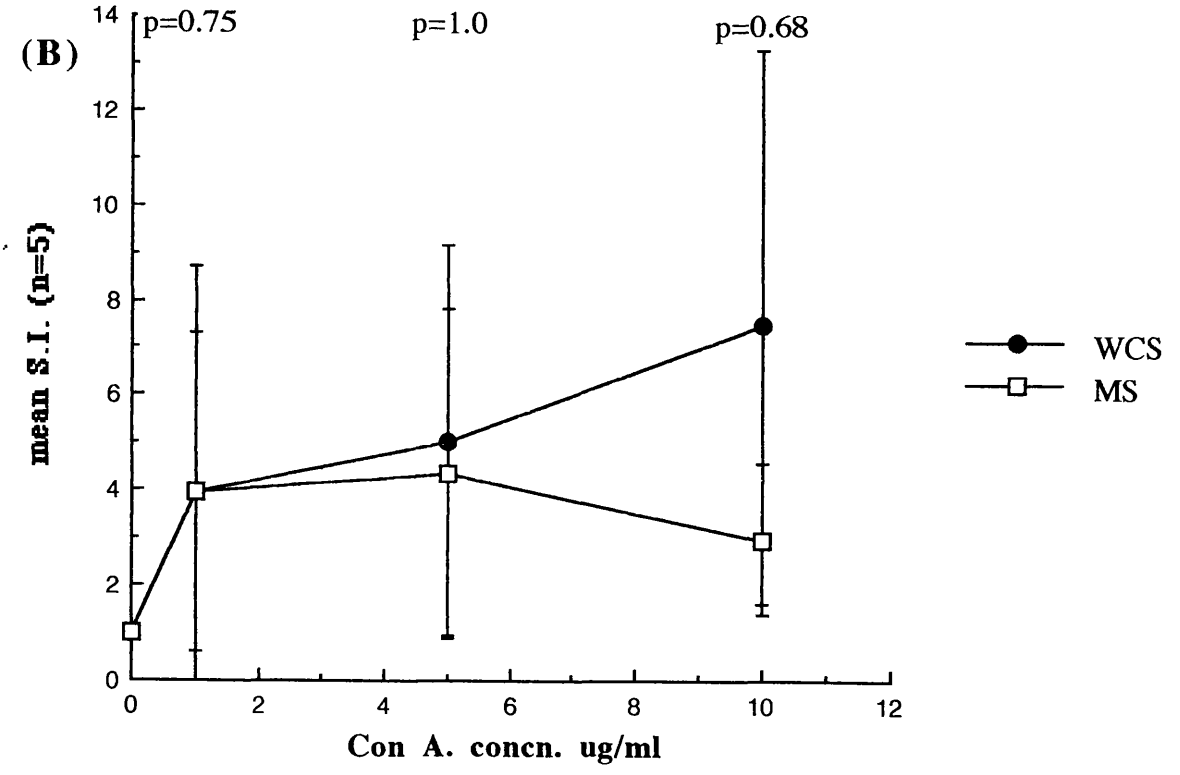
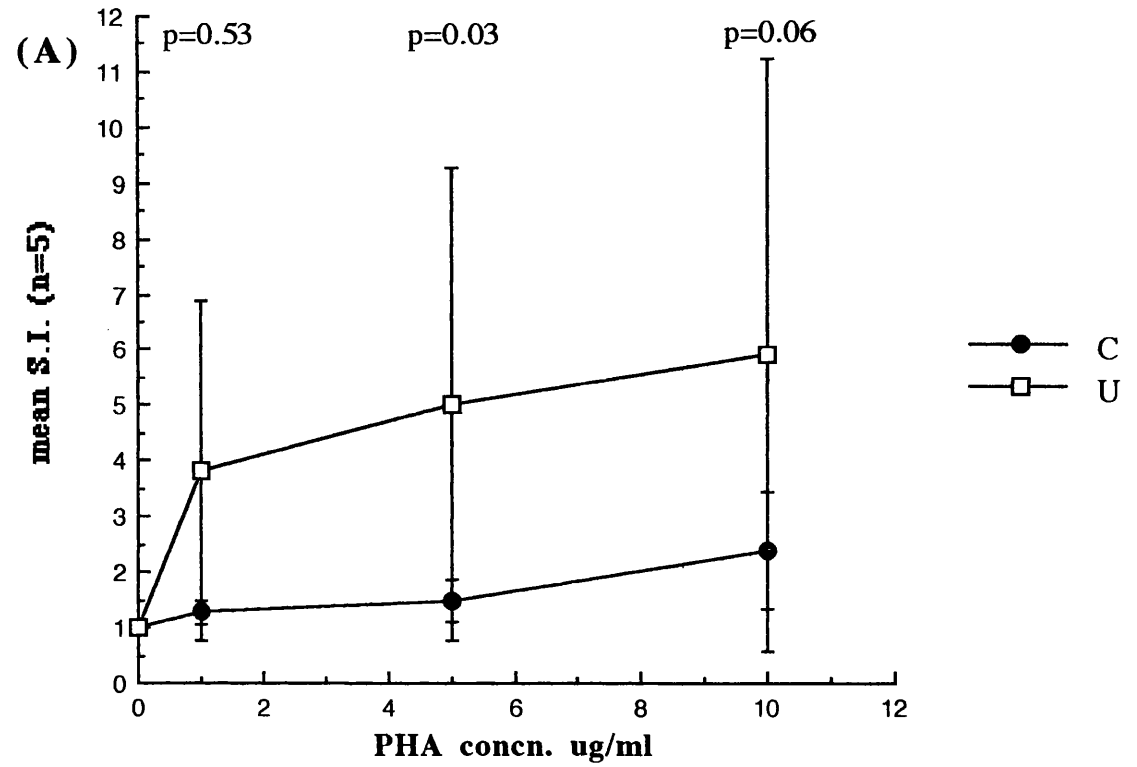


Figure 22. Graphical representation of the data illustrating the different responses of PBLs from A) chronically infected (C) and B) uninfected (U) woodchucks to Con A. PBLs were incubated in Clicks medium with either 10% WCS or 10% MS and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.



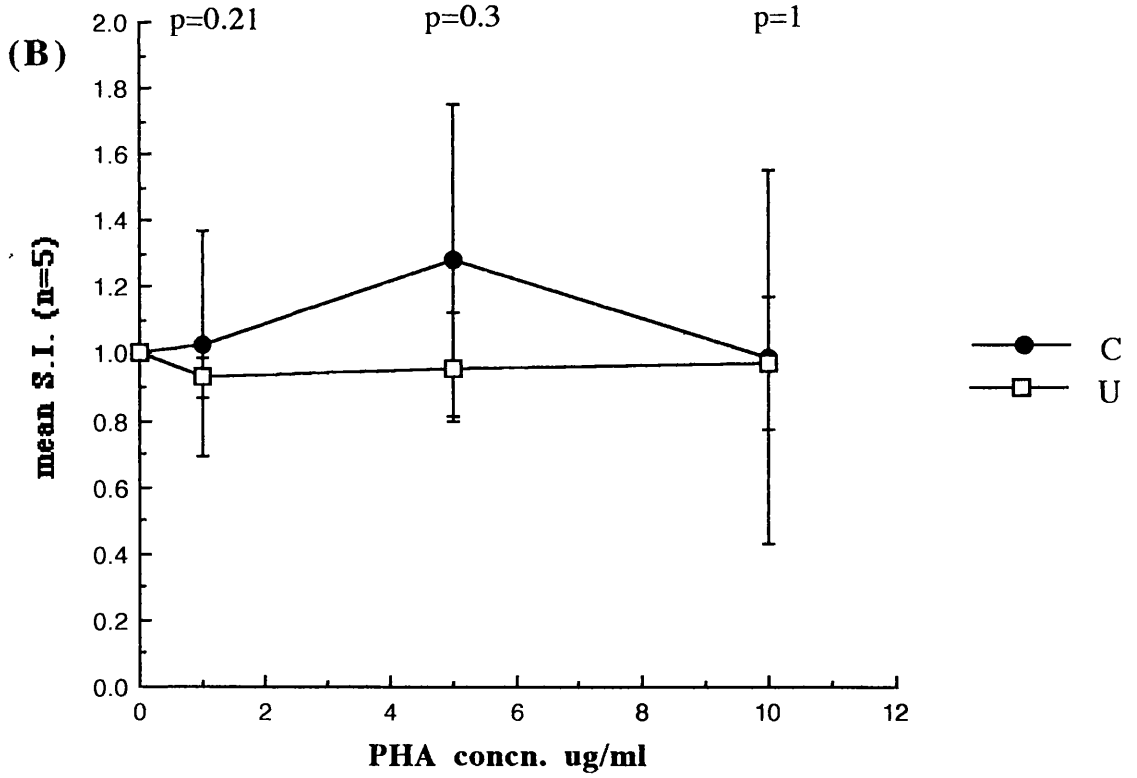
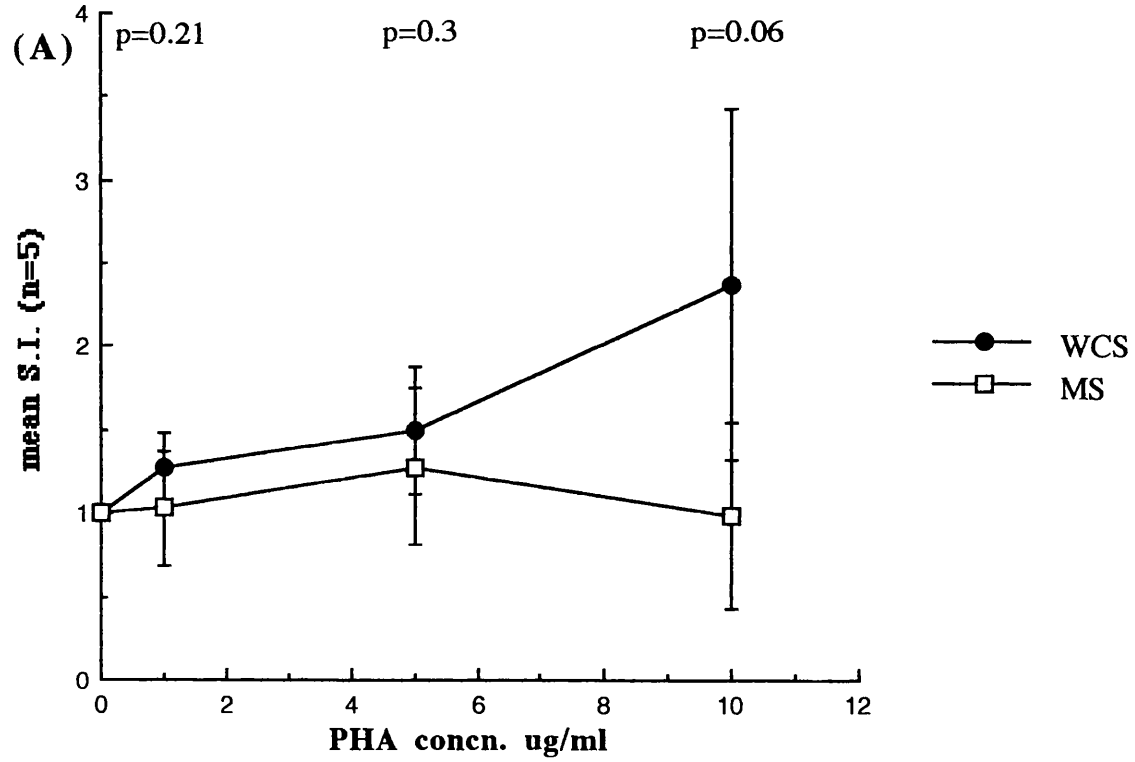


Figure 23. Graphical representation of the data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to PHA. PBLs were incubated in Clicks medium with A) 10% WCS and B) 10% MS and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.



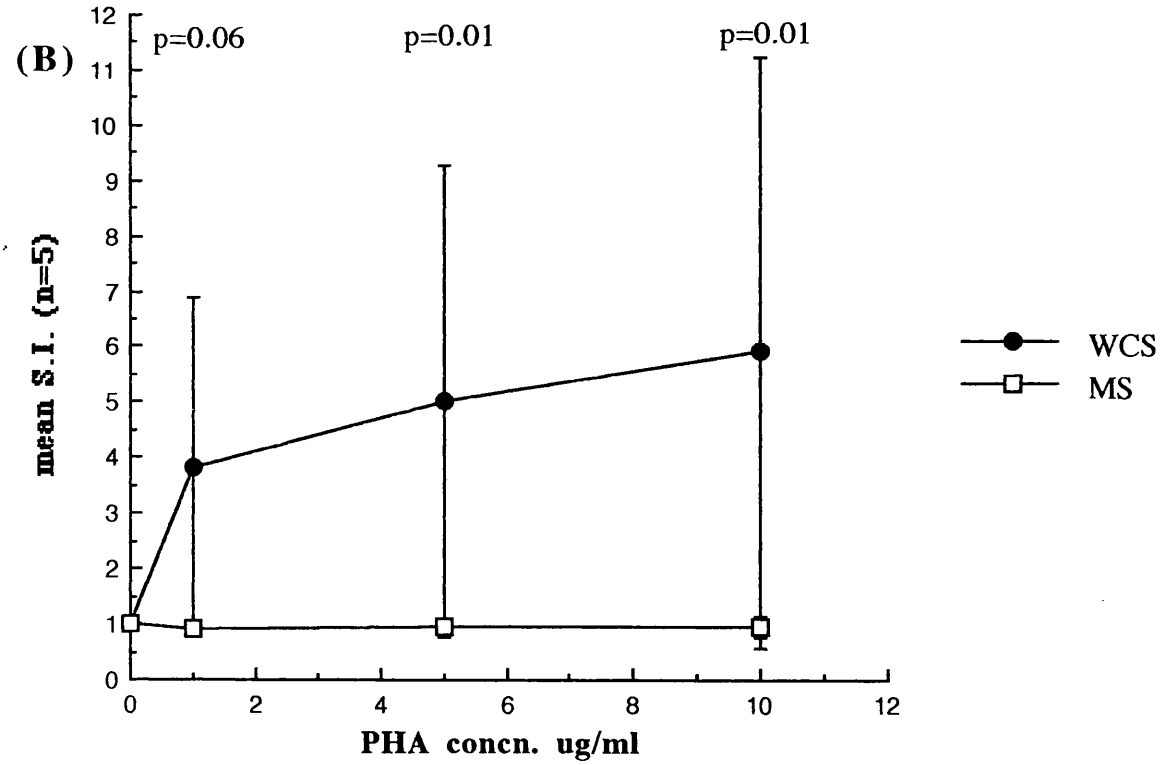
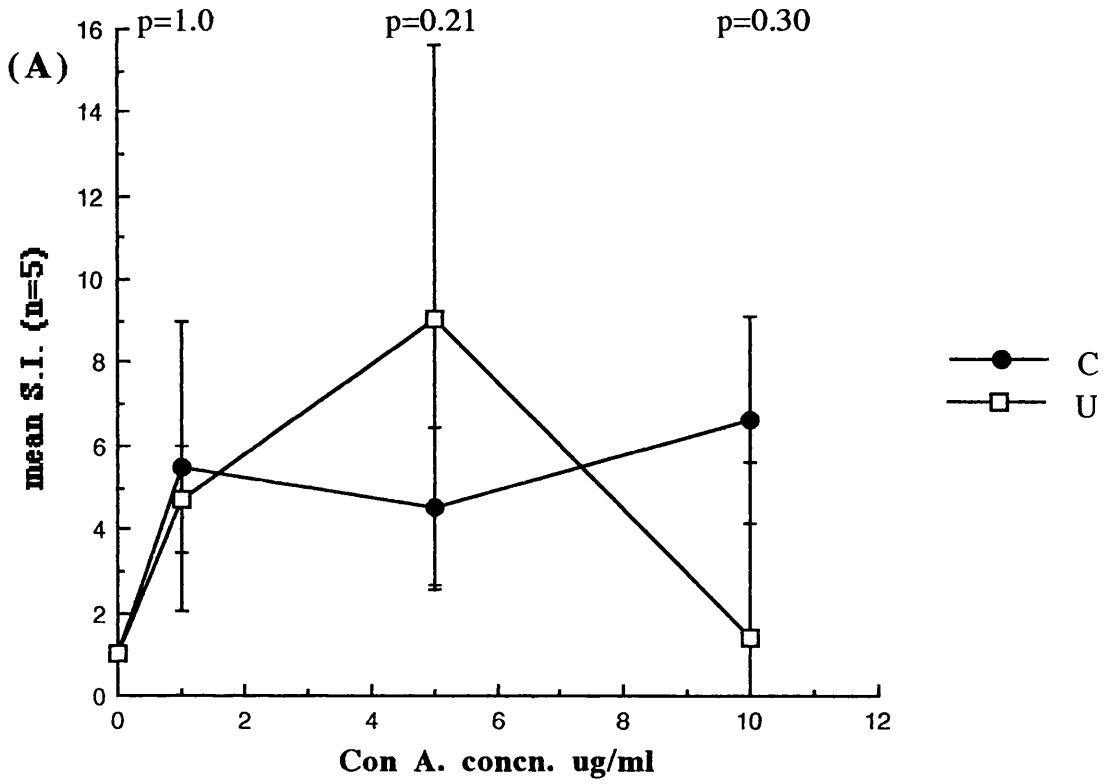


Figure 24. Graphical representation of the data comparing the different responses of PBLs from A) chronically infected (C) and B) uninfected (U) woodchucks to PHA. PBLs were incubated in Clicks medium with either 10% WCS or 10% MS and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.

Woodchuck (Number)	Basal Incorporation (Mean cpm ± S.E.M.)			
	0.5% MS		0.5% WCS	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Chronically Infected (5)	558 ± 655	268 ± 347	72 ± 23	72 ± 23
Uninfected (5)	43 ± 30	43 ± 30	61 ± 32	61± 32
p Value	0.53	0.03	0.53	0.06

Table 11. Basal level of incorporation of Udr into unstimulated woodchuck PBLs from chronically infected and uninfected animals incubated in Clicks medium supplemented with 0.5% serum from mouse and woodchuck. Mann-Whitney U-test p values <0.05 were considered significant.

There was no significant difference between chronically infected and uninfected animals using 0.5% WCS or 0.5% MS with Con A (figure 25). There was also no significant difference between uninfected animals using either supplement; however, there was a significant difference between the two serum supplements using 10ug/ml Con A in chronically infected animals (figure 26). There was a significant difference between chronically infected and uninfected animals using 0.5% WCS in response to PHA but none using 0.5% mouse serum (figure 27). There was also no significant difference between 0.5% WCS or mouse serum in chronically infected animals but in uninfected animals a significant difference was observed using 1 and 5ug/ml PHA (figure 28). (Mean cpm data in Appendix II, tables 19-22)



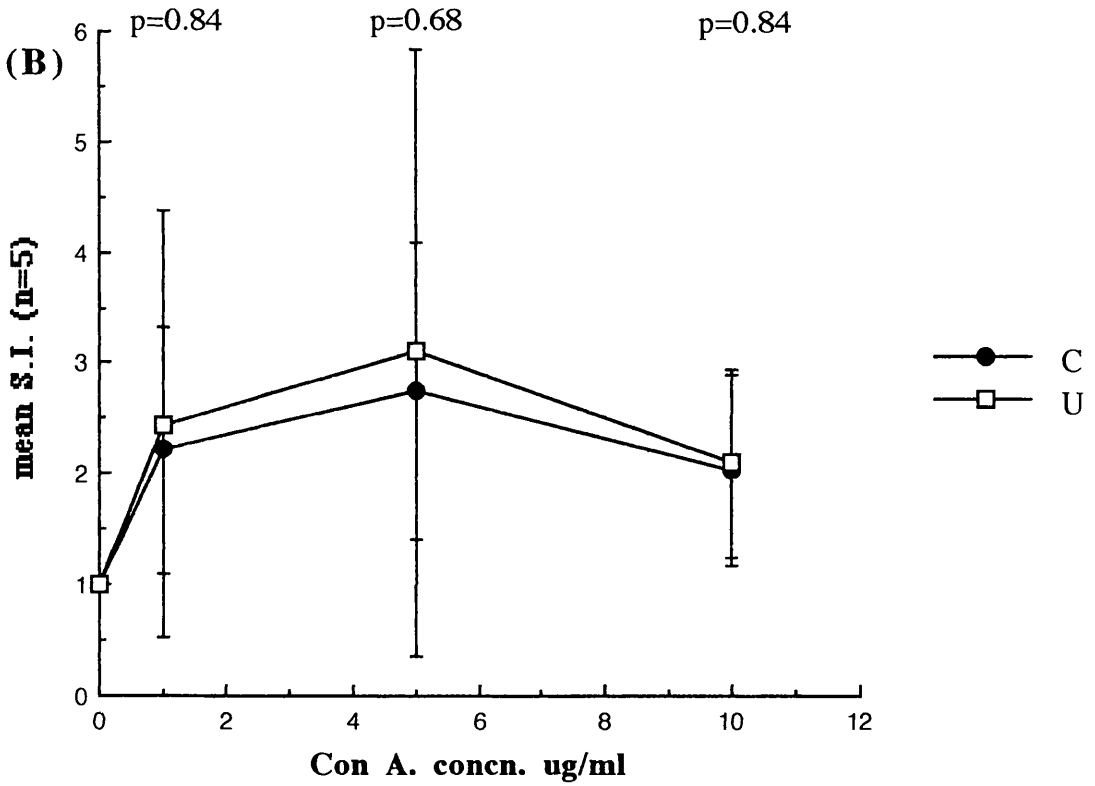
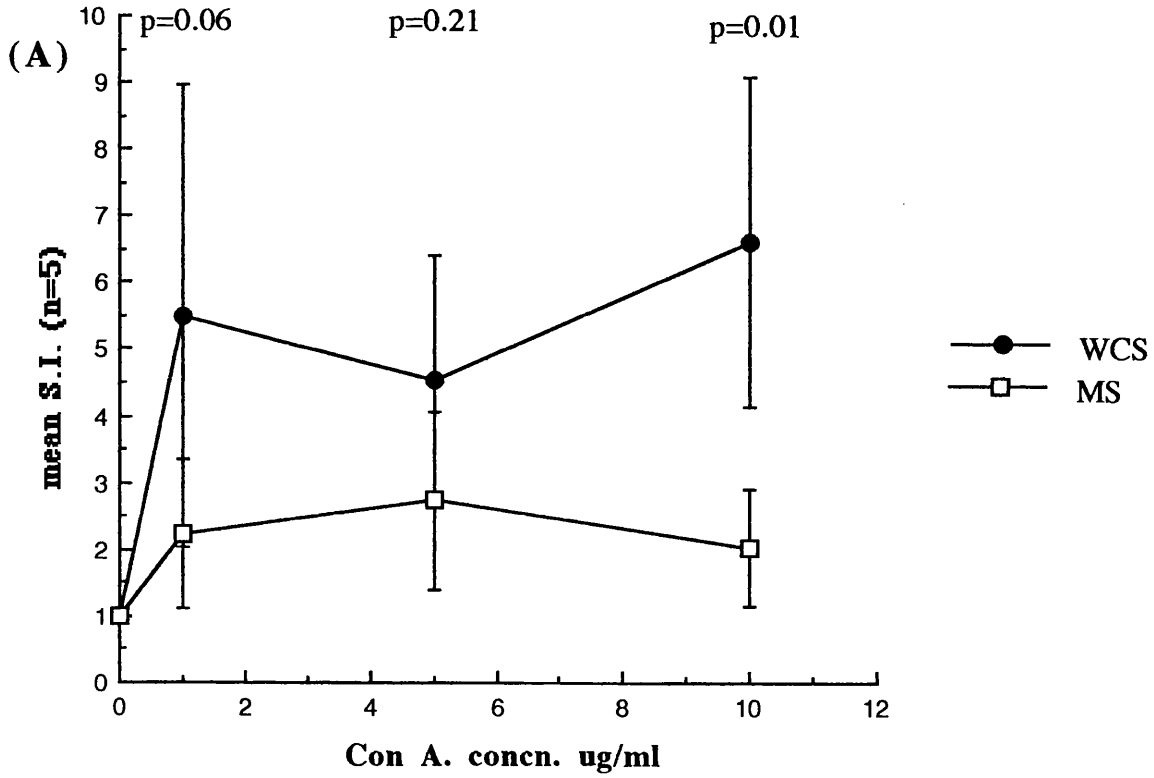


Figure 25. Graphical representation of the data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to Con A. PBLs were incubated in Clicks medium with A) 0.5% WCS and B) 0.5% MS and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.



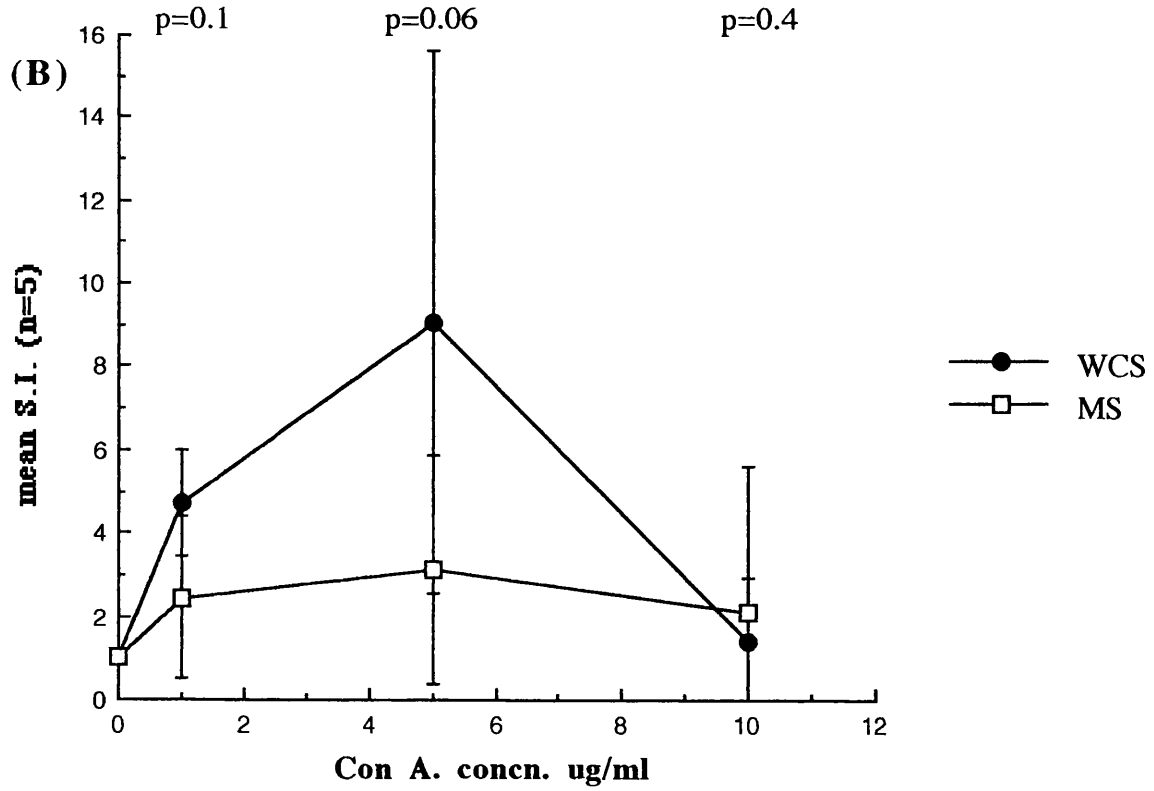
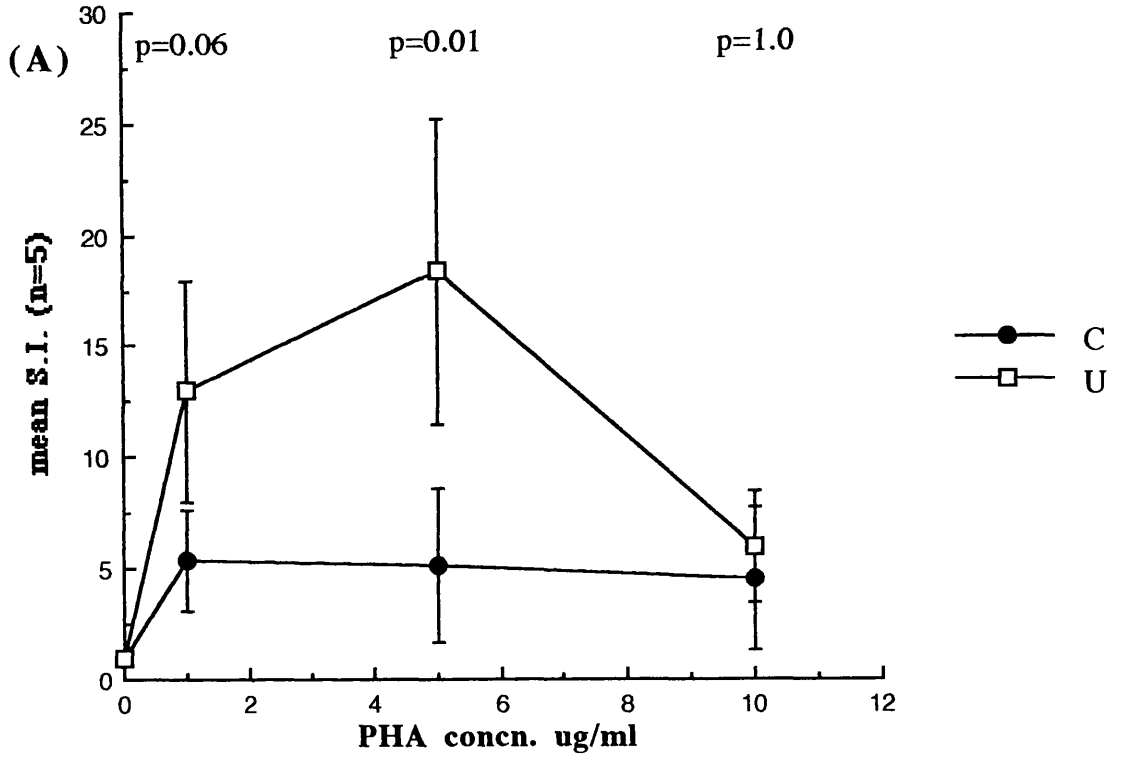


Figure 26. Graphical representation of the data comparing the different responses of PBLs from A) chronically infected (C) and B) uninfected (U) woodchucks to Con A. PBLs were incubated in Clicks medium with either 0.5% WCS or 0.5% MS and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.



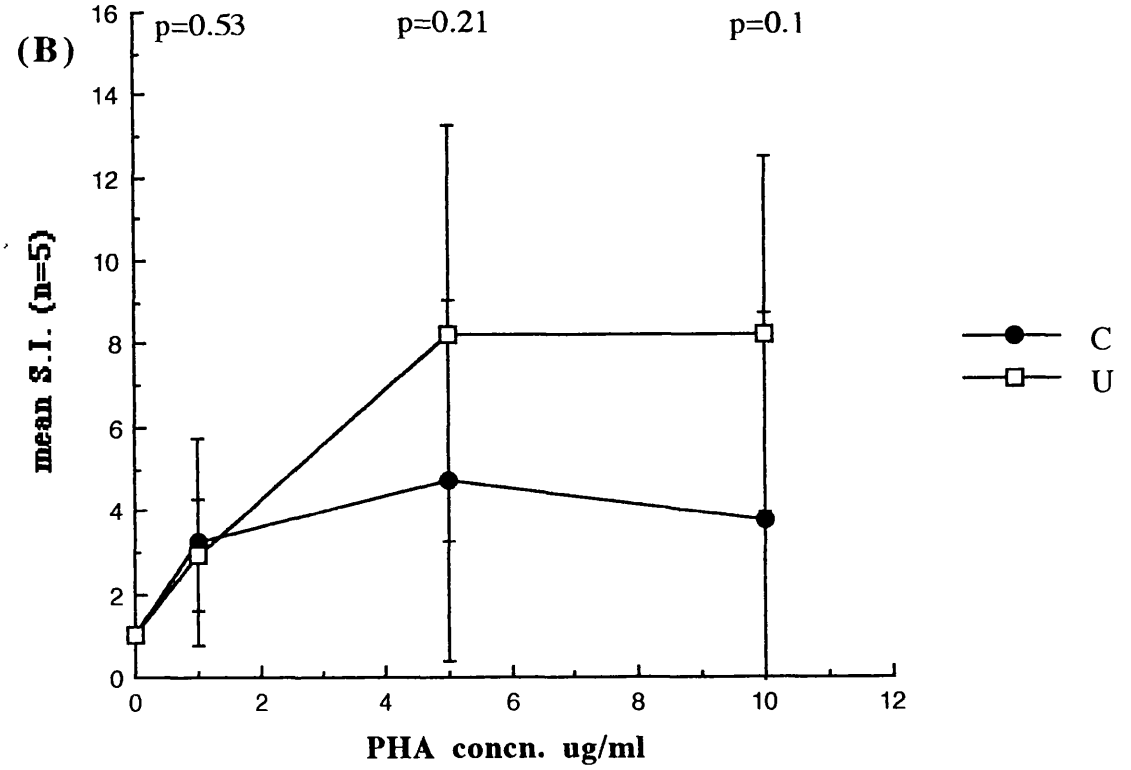
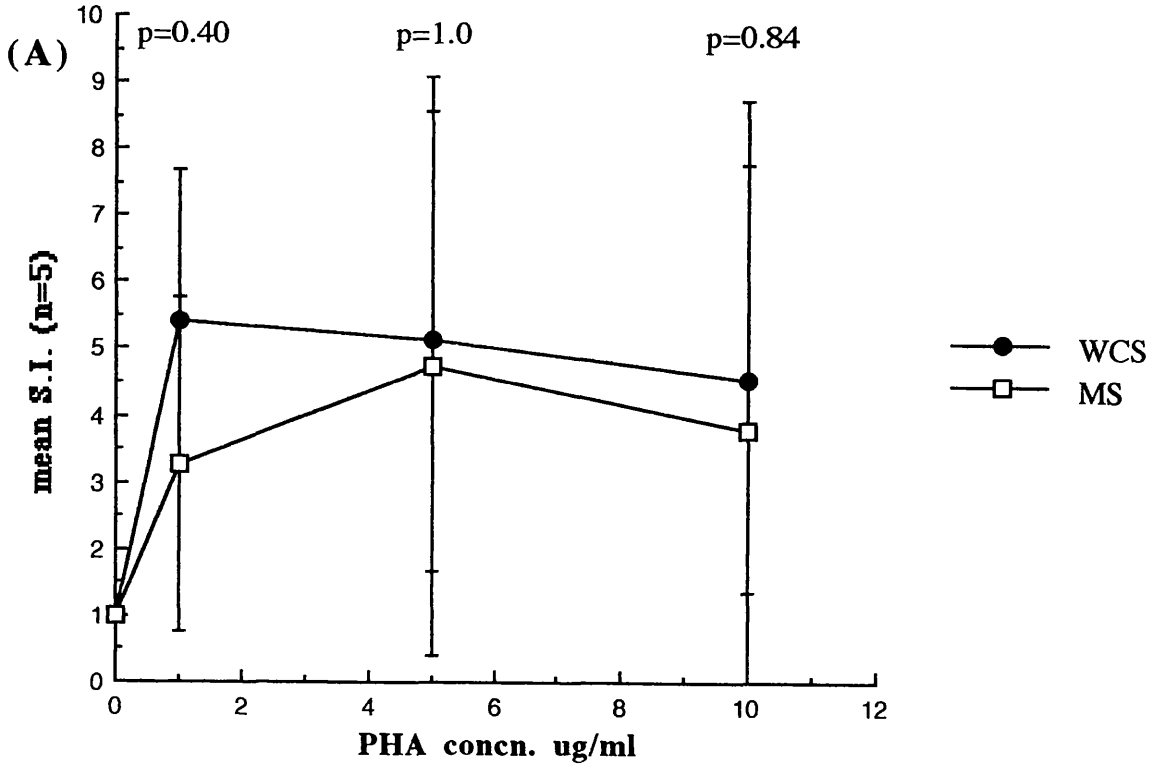


Figure 27. Graphical representation of the data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to PHA. PBLs were incubated in Clicks medium with A) 0.5% WCS and B) 0.5% MS and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.



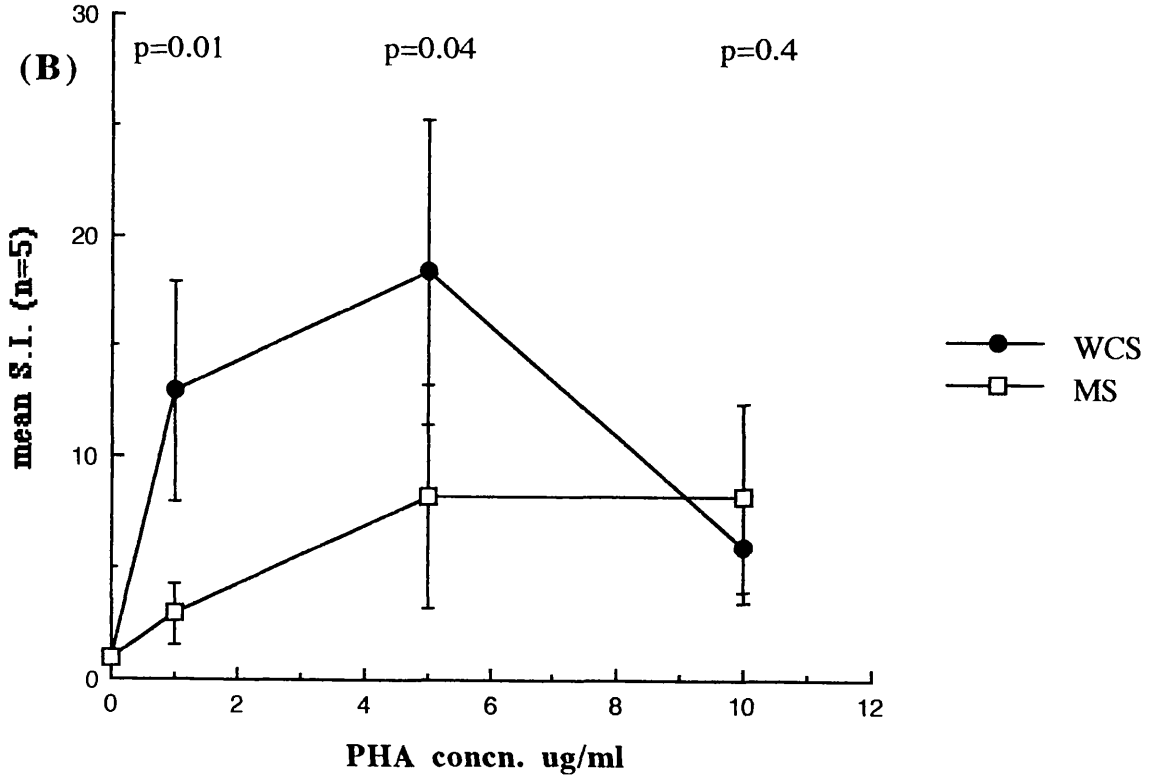


Figure 28. Graphical representation of the data comparing the different responses of PBLs from A) chronically infected (C) and B) uninfected (U) woodchucks to PHA. PBLs were incubated in Clicks medium with either 0.5% WCS or 0.5% MS and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.

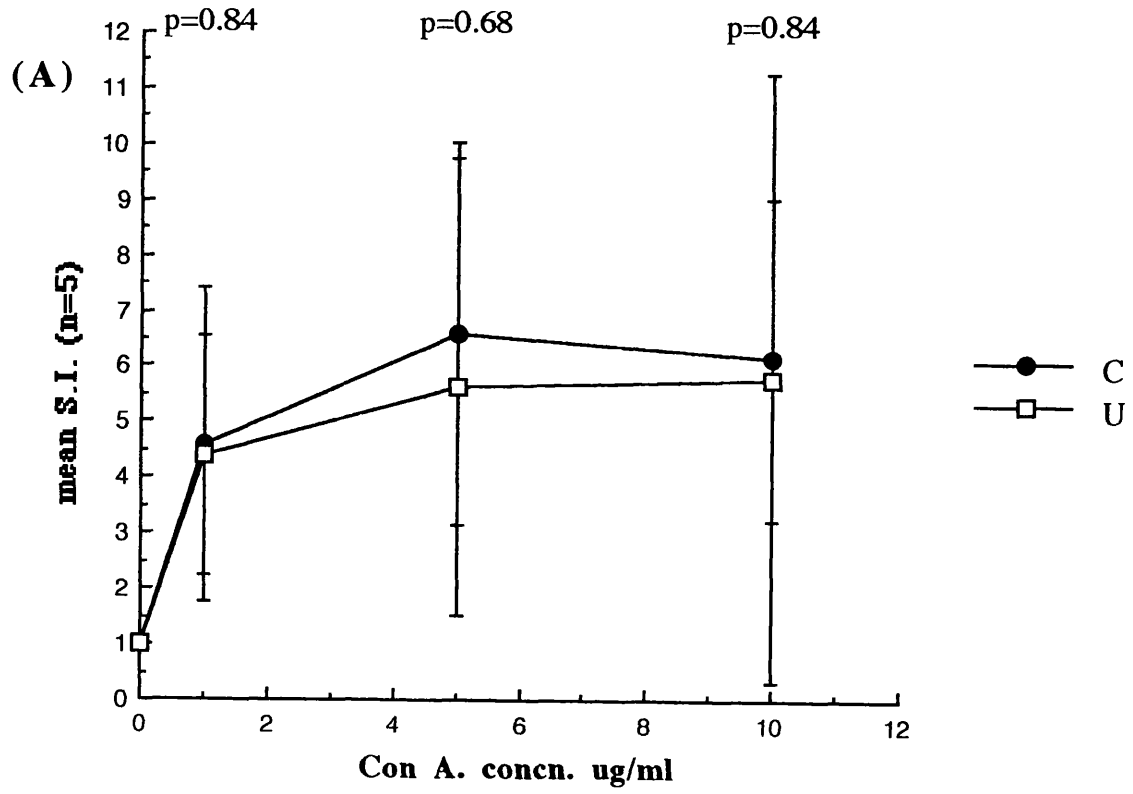
2.4.7. The Effect of Addition of 2-ME to the Culture Medium on the Proliferation of Woodchuck PBLs

Clicks medium supplemented with 0.5% WCS was used to test the effectiveness of 2-ME. Table 12 summarises the background level of incorporation in unstimulated PBLs from both chronically infected and uninfected animals.

Woodchuck (Number)	Basal Incorporation (Mean cpm ± S.E.M.)			
	Exp. 1		Exp. 2	
	+ 2-ME	- 2-ME	+ 2-ME	- 2-ME
Chronically Infected (5)	160 ± 84	72 ± 23	73 ± 84	72 ± 23
Uninfected (5)	116 ± 62	61 ± 32	116 ± 62	61 ± 32
p Value	0.53	0.53	0.53	0.53

Table 12. Basal level of incorporation of Udr into unstimulated woodchuck PBLs from chronically infected and uninfected animals incubated in Clicks medium supplemented with 0.5% WCS in the presence and absence of 2-ME.. Mann-Whitney U-test p values <0.05 were considered significant.

There was no significant difference between the S.I. values from chronically infected and uninfected animals using Con A in the presence or absence of 2-ME (figure 29). There was also no significant difference in the S.I. values obtained with and without 2-ME in chronically infected animals; this was also true for uninfected animals (figure 30). There was no significant difference in the S.I. values obtained using PHA between chronically infected and uninfected animals in the presence of 2-ME, however, in the absence of 2-ME a significant difference was observed using 1 and 5ug/ml PHA (figure 31). There was no significant difference in the S.I. values in the presence and absence of 2-ME in both chronically infected and uninfected animals (figure 32). However, the S.I. values were higher in chronically infected animals in the presence of 2-ME. (Mean cpm data in Appendix II, tables 23-26)



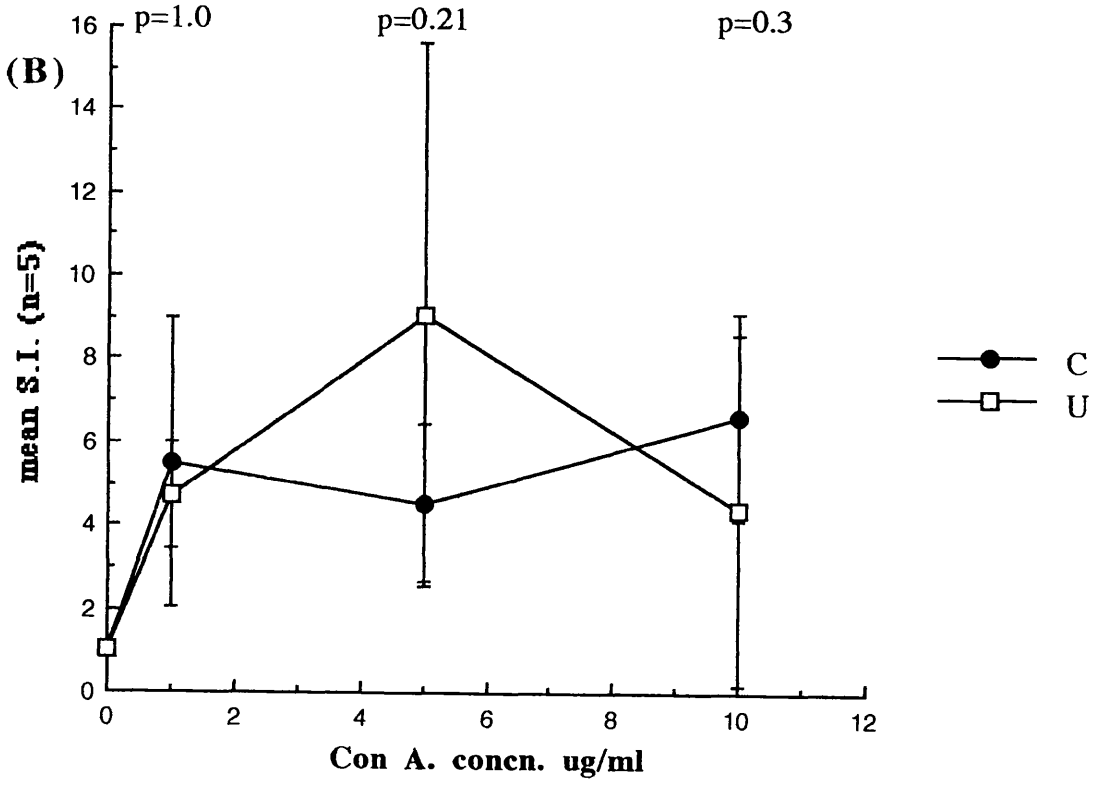


Figure 29. Graphical representation of the data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to Con A. PBLs were incubated in Clicks medium with 0.5% WCS in A) the presence of 2-ME B) the absence of 2-ME and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.

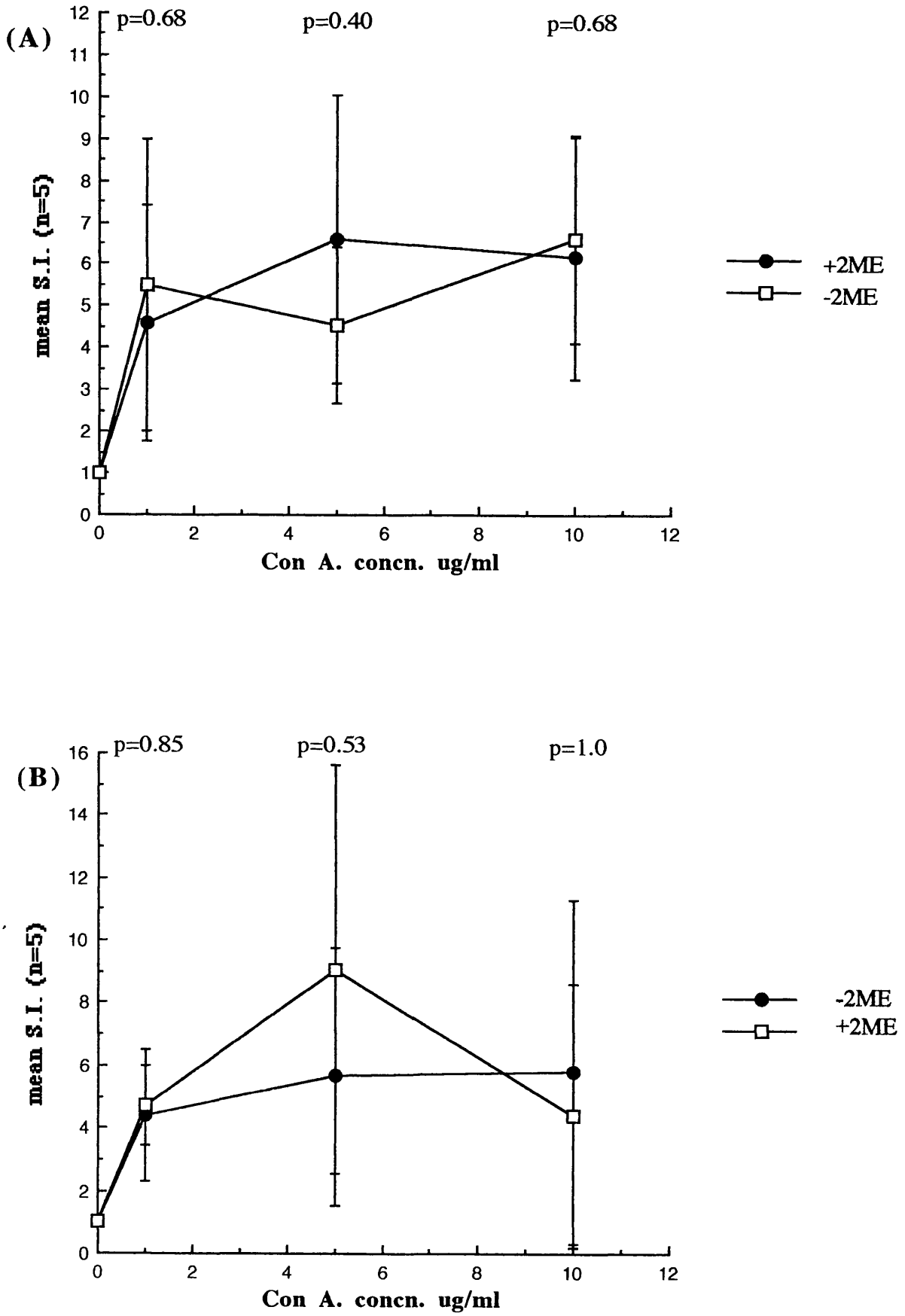
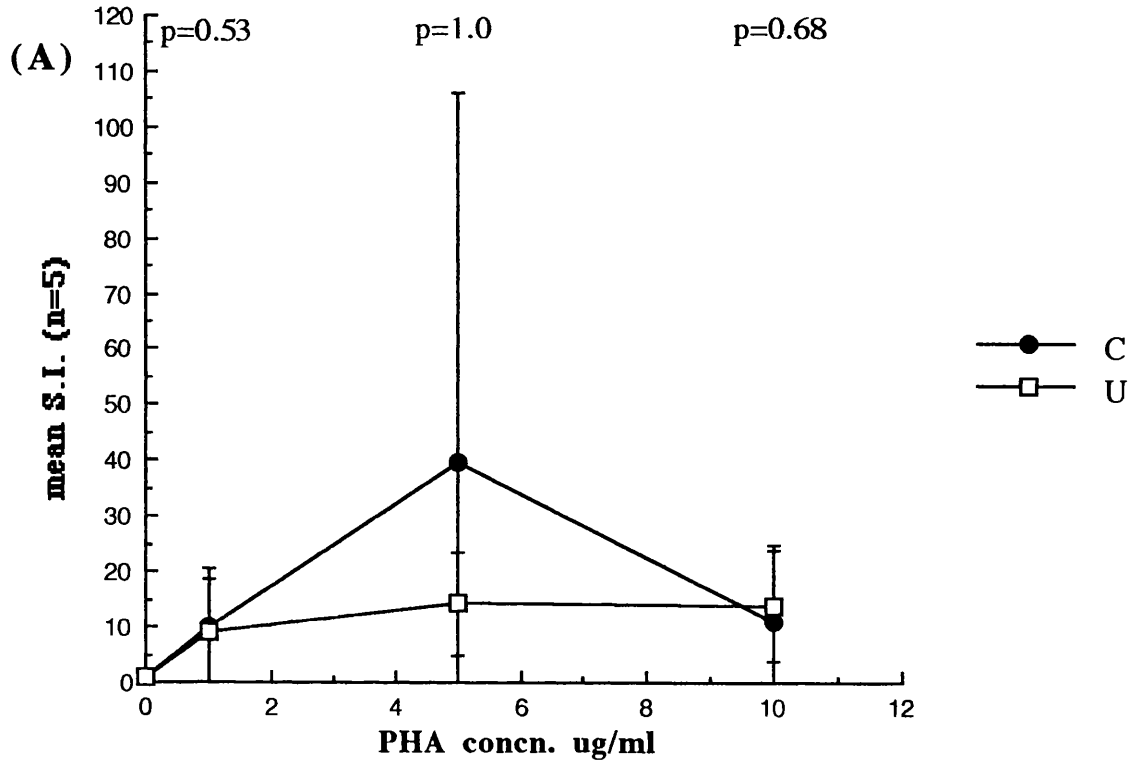


Figure 30. Graphical representation of the data comparing the different responses of PBLs from A) chronically infected (C) and B) uninfected (U) woodchucks to Con A in the presence or absence of 2-ME. PBLs were incubated in Clicks medium with 0.5% WCS and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.



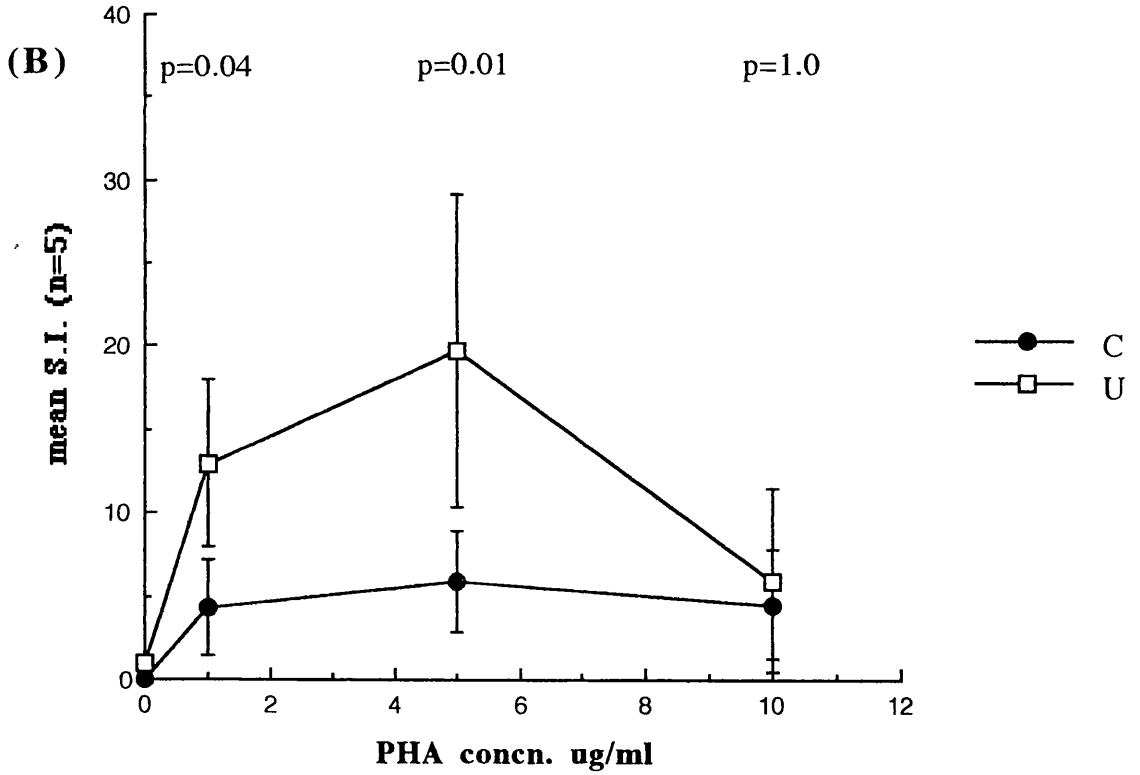


Figure 31. Graphical representation of the data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to PHA. PBLs were incubated in Clicks medium with 0.5% WCS in A) the presence of 2-ME B) the absence of 2-ME and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.

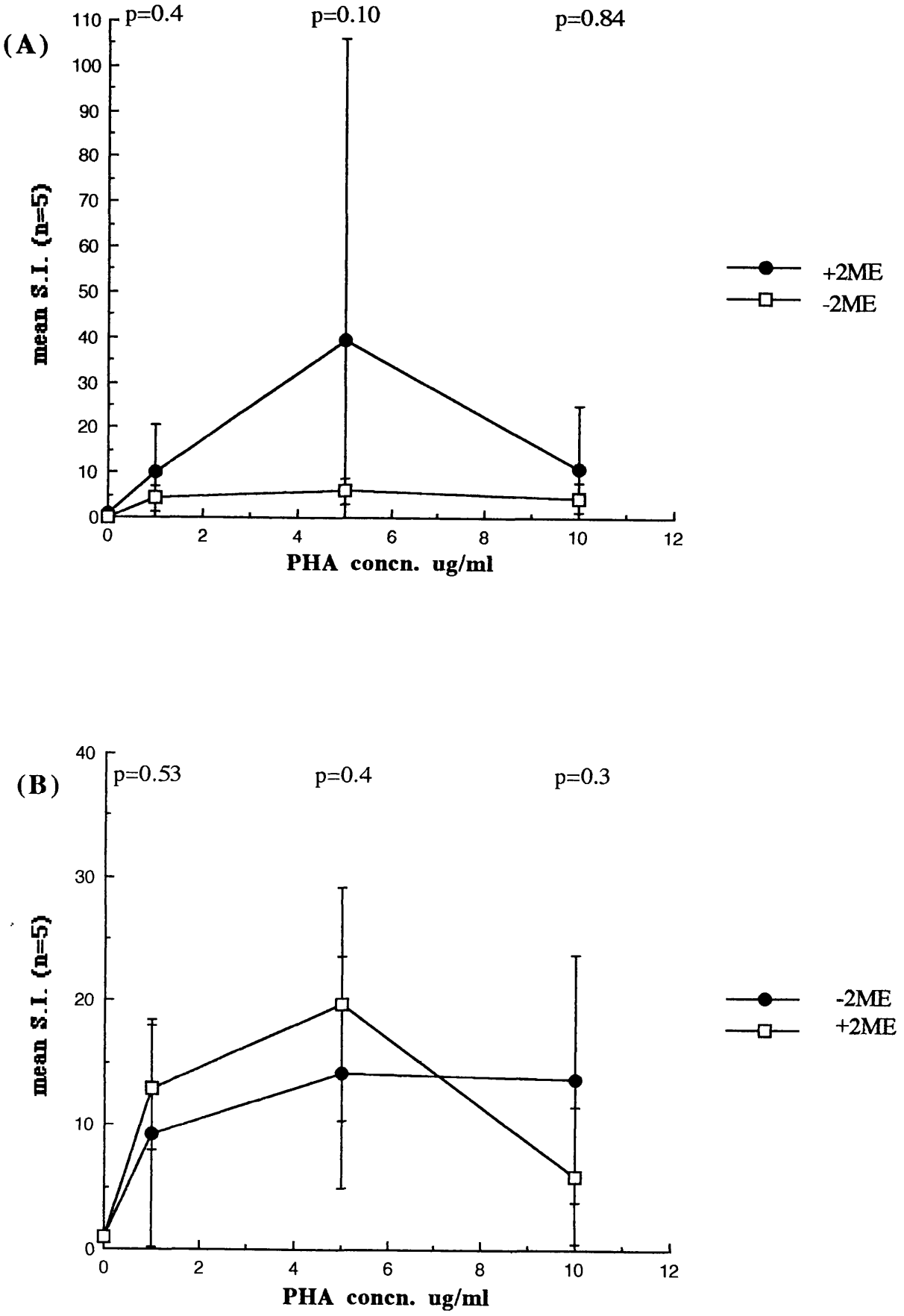


Figure 32. Graphical representation of the data comparing the different responses of PBLs from A) chronically infected (C) and B) uninfected (U) woodchucks to PHA in the presence or absence of 2-ME. PBLs were incubated in Clicks medium with 0.5% WCS and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.

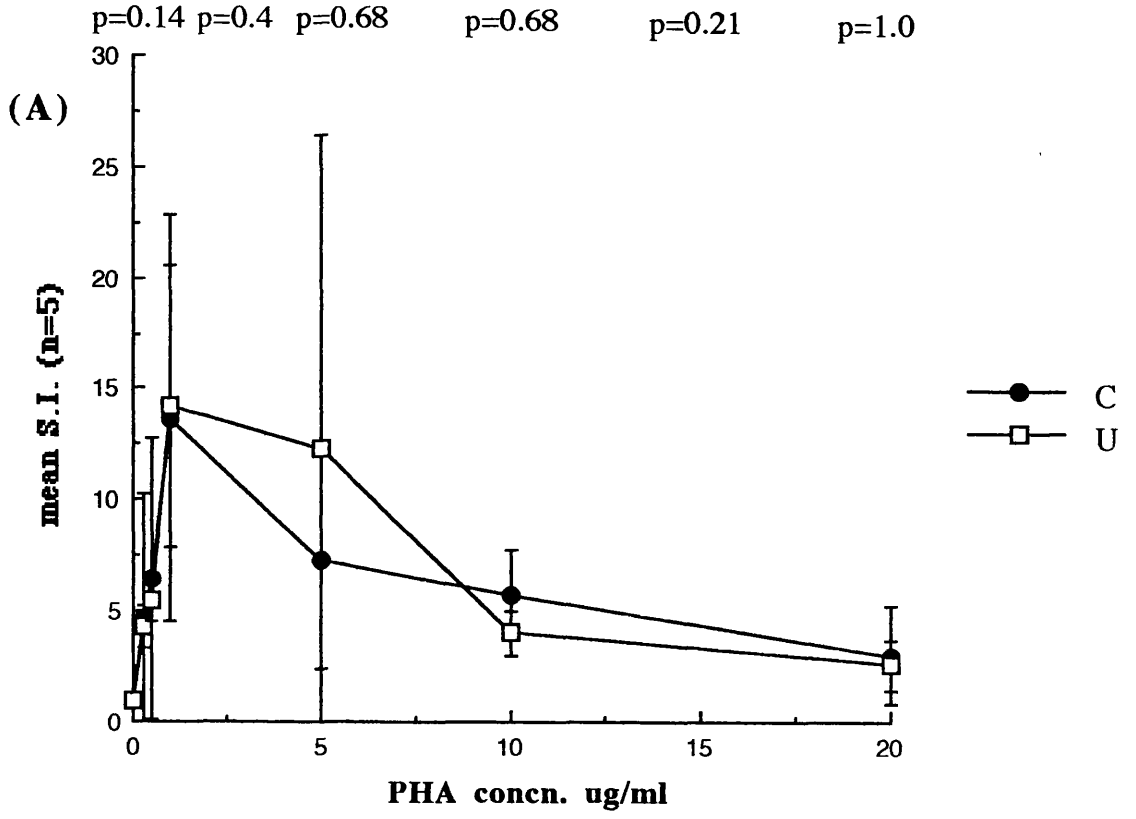
2.4.8. The Effect of Different Mitogens on the Proliferation of Woodchuck PBLs

There is a significant difference between the background level of radiolabel incorporation in chronically infected and uninfected animals. Table 13 summarises the results obtained.

Woodchuck (Number)	Basal Incorporation (Mean cpm ± S.E.M.)	
	Exp. 1	Exp. 2
Chronically Infected (5)	421 ± 182	421 ± 182
Uninfected (5)	98 ± 48	98 ± 48
p Value	0.01	0.01

Table 13. Basal level of incorporation of Udr into unstimulated woodchuck PBLs from chronically infected and uninfected animals incubated in Clicks medium supplemented with 0.5% WCS and 2-ME for 4 days. Mann-Whitney U-test p values <0.05 were considered significant.

There was no significant difference between the S.I. values obtained using PHA or Con A in chronically infected and uninfected animals (figure 33). There was also no significant difference between the S.I. values obtained using PHA and Con A in chronically infected animals (figure 34). However, at concentrations of 1, 10 and 20ug/ml there was a significant difference in the S.I. values obtained using PHA and Con A in uninfected animals (figure 34). (Mean cpm data in Appendix II, tables 27-30)



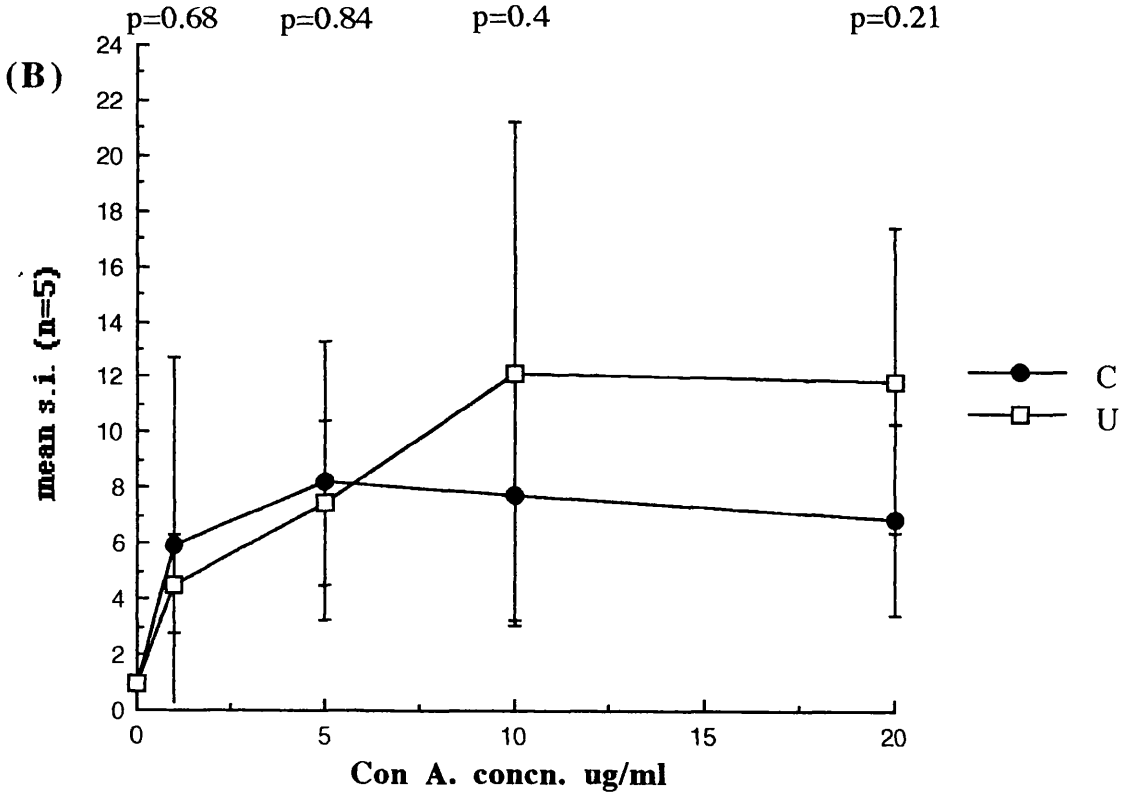
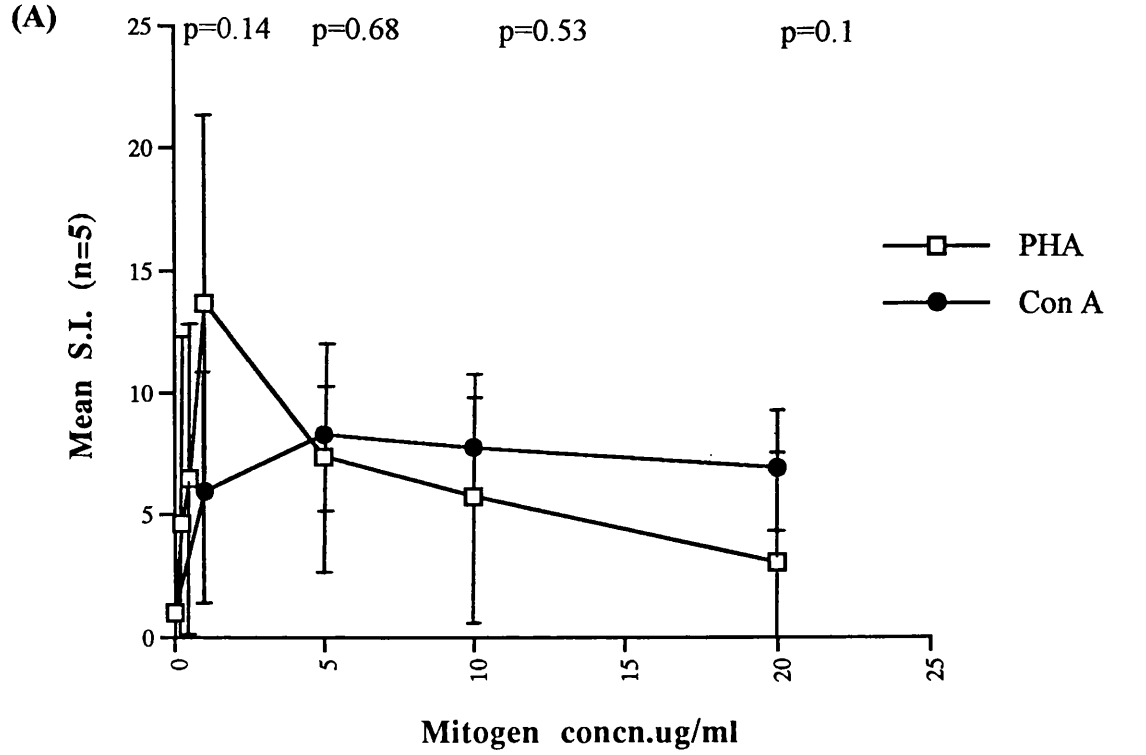


Figure 33. Graphical representation of the data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to mitogen. PBLs were incubated in Clicks medium with 0.5% WCS in the presence of A) PHA B) Con A and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.



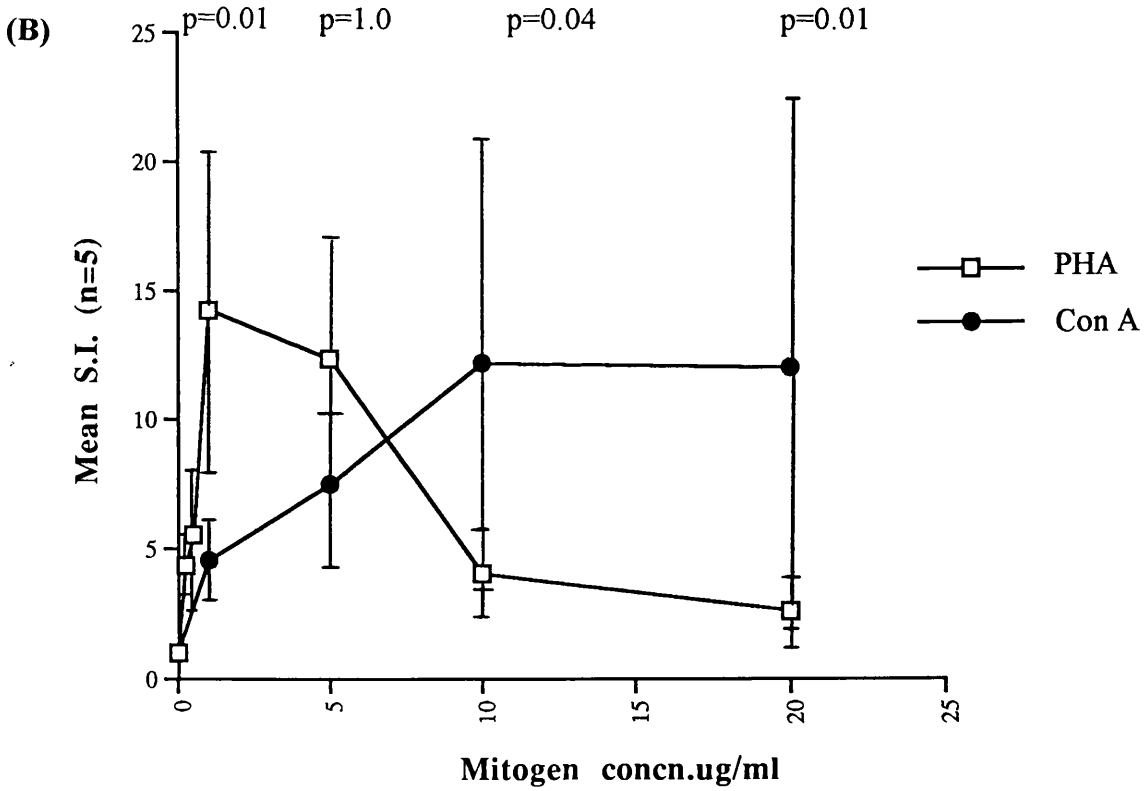


Figure 34. Graphical representation of the data comparing the different responses of PBLs from A) chronically infected (C) and B) uninfected (U) woodchucks to both PHA and Con A. PBLs were incubated in Clicks medium supplemented with 0.5% WCS and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.

2.4.9. The Effect of Increasing the Incubation Time on the Proliferation of Woodchuck PBLs

There was a significant difference in the background incorporation of Udr between chronically infected and uninfected animals after 4 days but none after 7 days of incubation. Table 14 summarises the results obtained.

Woodchuck (Number)	Basal Incorporation (Mean cpm ± S.E.M.)	
	4 days	7 days
Chronically Infected (5)	421 ± 182	48 ± 31
Uninfected (5)	98 ± 48	25 ± 10
p Value	0.01	0.29

Table 14. Basal level of incorporation of Udr into unstimulated woodchuck PBLs from chronically infected and uninfected animals incubated in Clicks medium supplemented with 0.5% WCS and 2-ME for 4 and 7 days. Mann-Whitney U-test p values <0.05 were considered significant.

There was no significant difference in the S.I. values obtained using PHA after 7 days of incubation in chronically infected and uninfected animals (figure 35). There was also no significant difference between the S.I. values obtained after 4 and 7 days in either chronically infected or uninfected animals (figure 36). However, S.I. values were lower after 7 days in chronically infected animals. (Mean cpm data in Appendix II, tables 27,28 and 31)

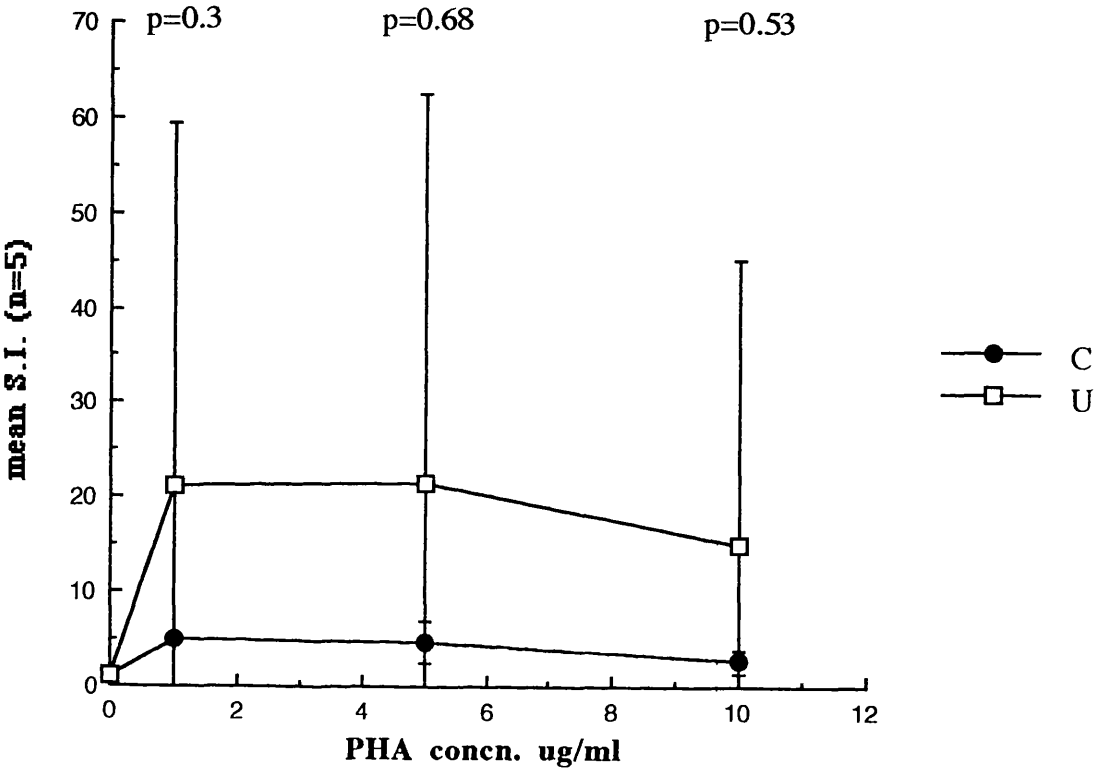
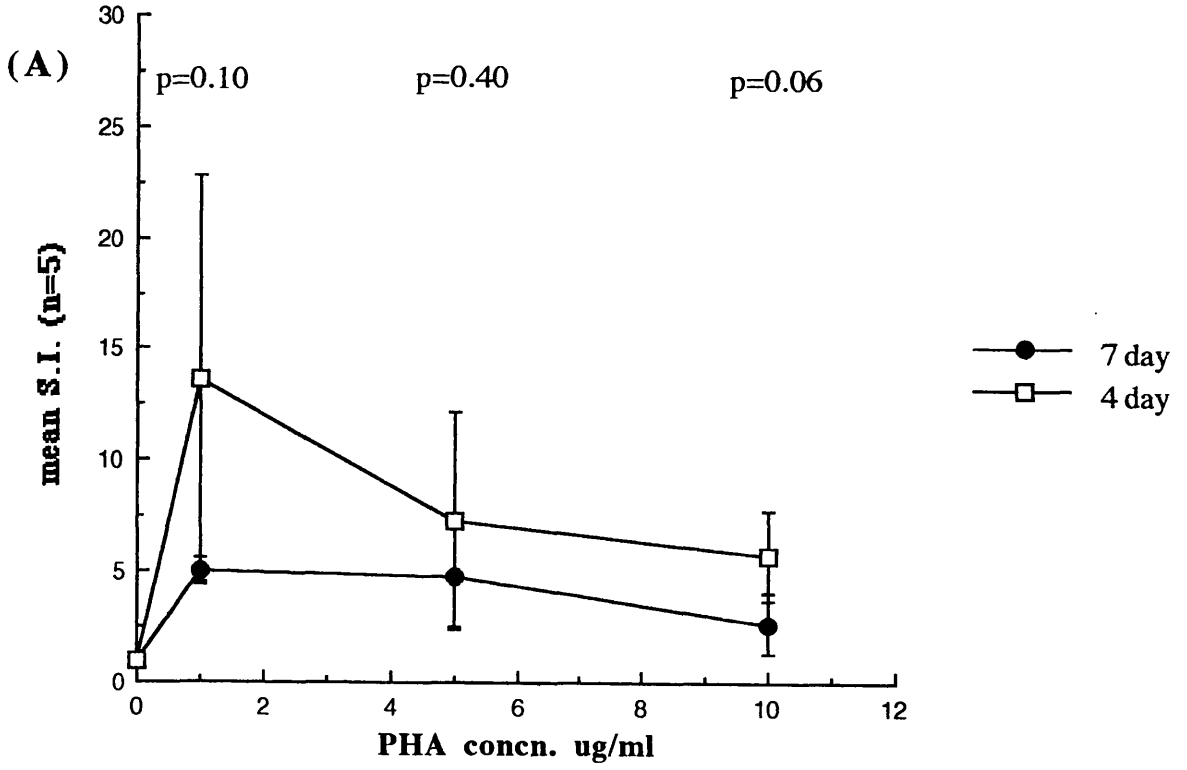


Figure 35. Graphical representation of the data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to PHA. PBLs were incubated in Clicks medium with 0.5% WCS and pulsed with Udr after 7 days. p Values < 0.05 were considered significant.



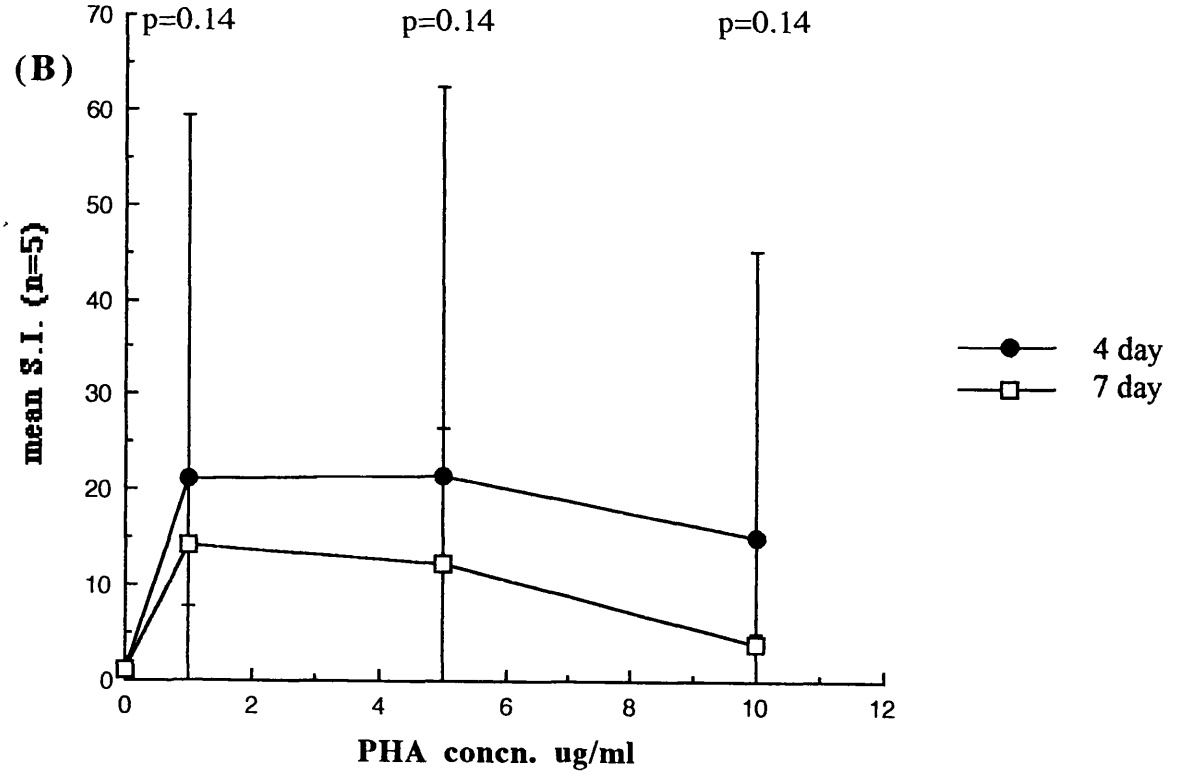


Figure 36. Graphical representation of the data comparing the different responses of PBLs from A) chronically infected (C) and B) uninfected (U) woodchucks to PHA after 4 and 7 days of incubation. PBLs were incubated in Clicks medium supplemented with 0.5% WCS and pulsed with Udr. p Values < 0.05 were considered significant.

2.4.10. The Effect of Substituting Different Radiolabels in the Culture Medium on the Proliferation of Woodchuck PBLs

There was no significant difference between the background level of incorporation between chronically infected and uninfected animals using Udr, Tdr or BrdU as label. Table 15 summarises the results obtained.

Woodchuck (Number)	Basal Incorporation (Mean cpm ± S.E.M.)		
	Uridine (Udr)	Thymidine (Tdr)	Bromo-deoxyuridine (BrdU)
Chronically Infected (5)	421 ± 182	175 ± 70	103 ± 84
Uninfected (5)	98 ± 48	184 ± 63	100 ± 42
p Value	0.01	0.83	0.67

Table 15. Basal level of incorporation of Udr, Tdr and BrdU into unstimulated woodchuck PBLs from chronically infected and uninfected animals incubated in Clicks medium supplemented with 0.5% WCS and 2-ME for 4 days. Mann-Whitney U-test p values <0.05 were considered significant.

There was no significant difference between the S.I values obtained using Tdr in chronically infected and uninfected animals in response to PHA (figure 37). There was a significant difference however, in the level of incorporation of Udr and Tdr in both chronically infected and uninfected animals (figure 38). There was no significant difference between the S.I. values obtained using BrdU in chronically infected and uninfected animals except at the highest concentration of mitogen (10ug/ml) (figure 39). However, there was a significant difference in the level of incorporation of Udr and BrdU in both chronically infected and uninfected animals at 1ug/ml and at 10ug/ml in uninfected animals only (figure 40). (Mean cpm data in Appendix II, tables 27, 28, 32 and 33)

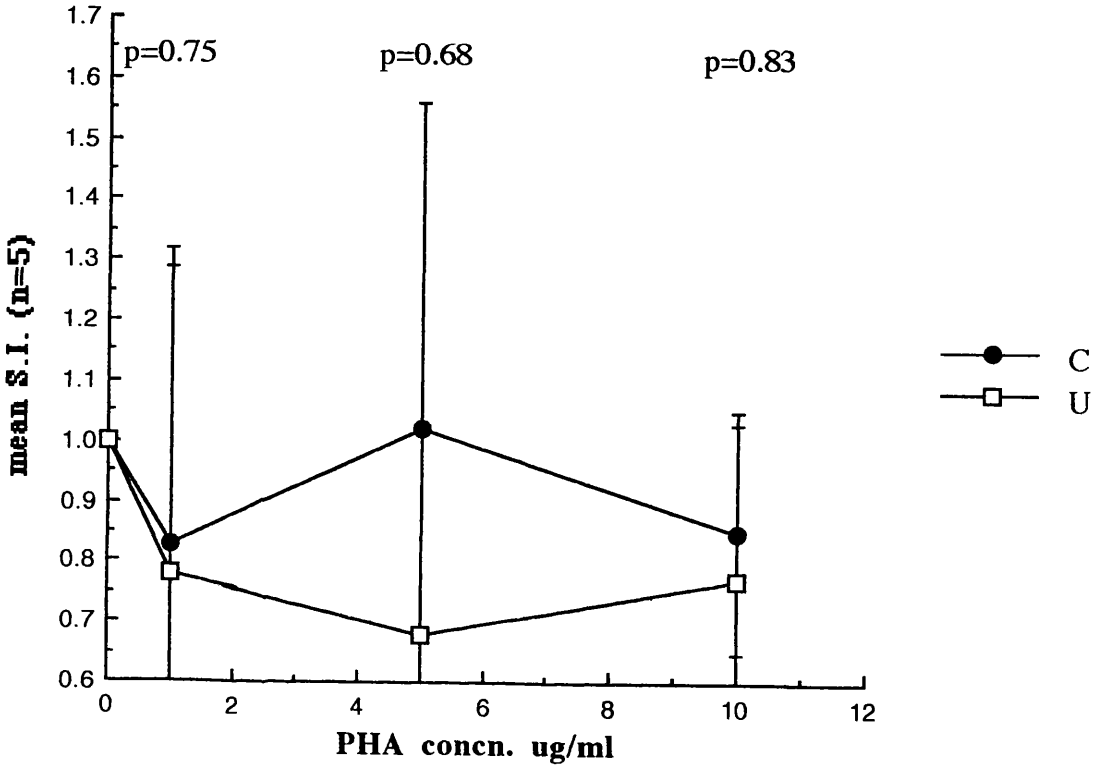
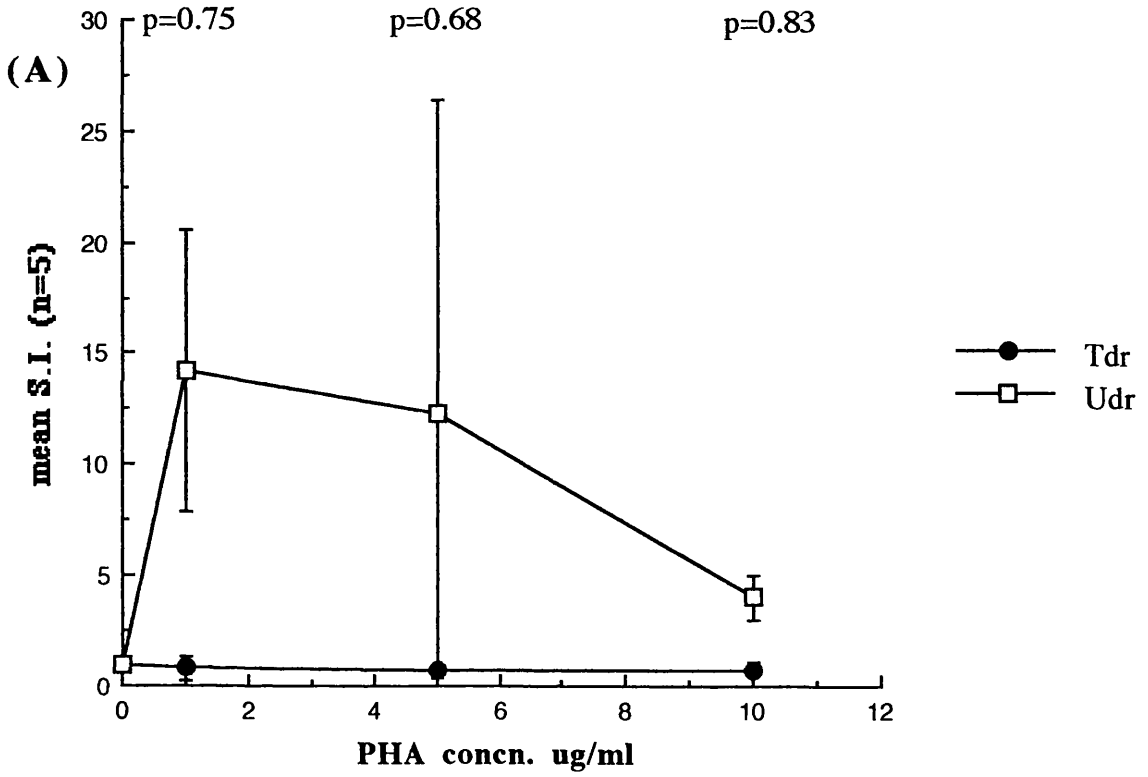


Figure 37. Graphical representation of the data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to PHA. PBLs were incubated in Clicks medium supplemented with 0.5% WCS and 2-ME and pulsed with Tdr after 4 days. p Values < 0.05 were considered significant.



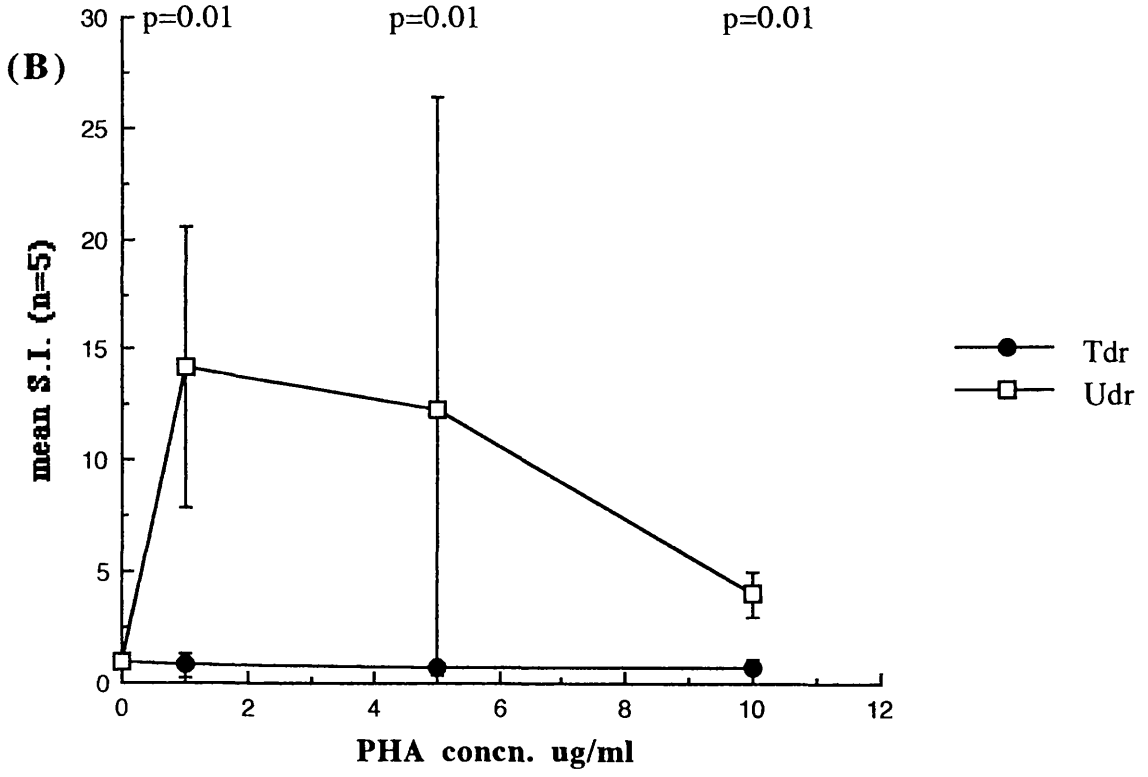


Figure 38. Graphical representation of the data comparing the incorporation of Udr and Tdr in PBLs from A) chronically infected (C) and B) uninfected (U) woodchucks after stimulation with PHA. PBLs were incubated in Clicks medium supplemented with 0.5% WCS and pulsed after 4 days. p Values < 0.05 were considered significant.

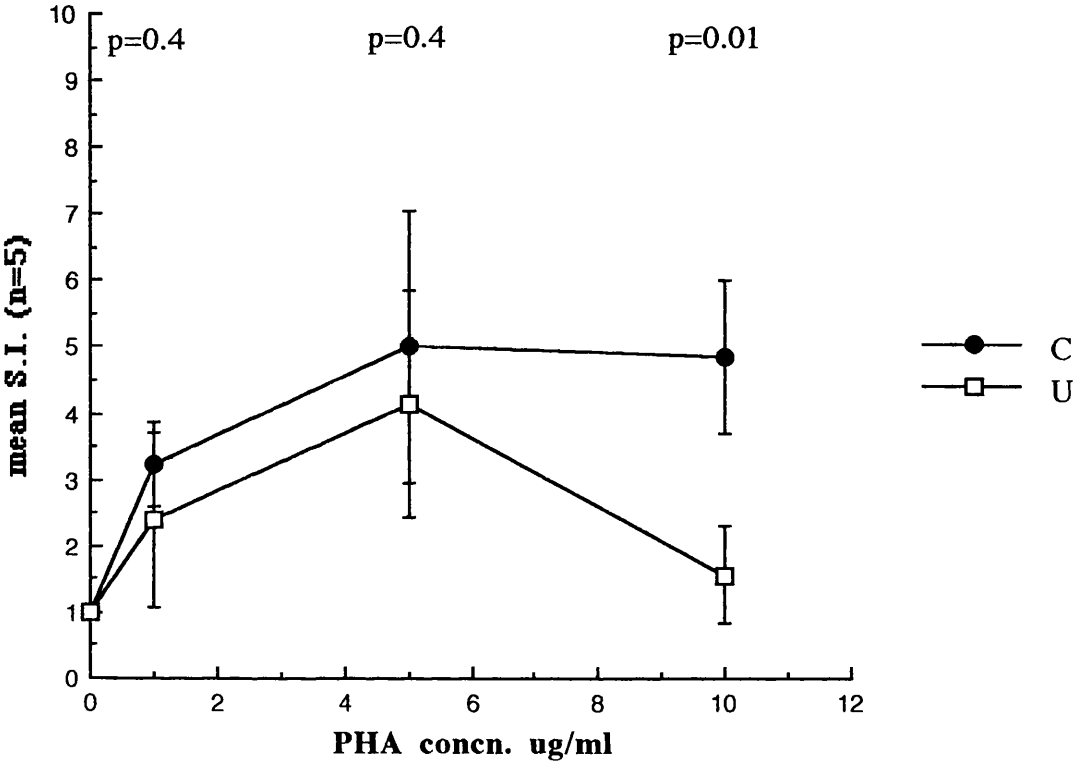
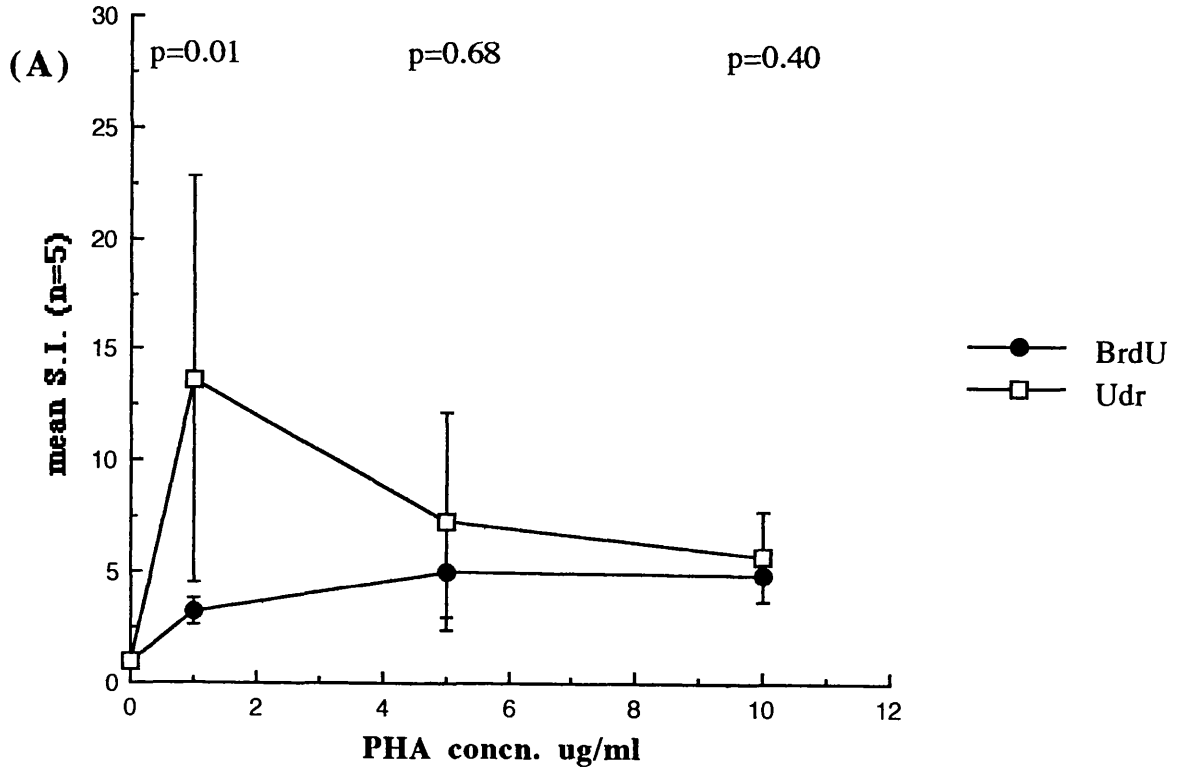


Figure 39. Graphical representation of the data illustrating the different responses of PBLs from chronically infected (C) and uninfected (U) woodchucks to PHA. PBLs were incubated in Clicks medium supplemented with 0.5% WCS and 2-ME and pulsed with BrdU after 4 days. p Values < 0.05 were considered significant.



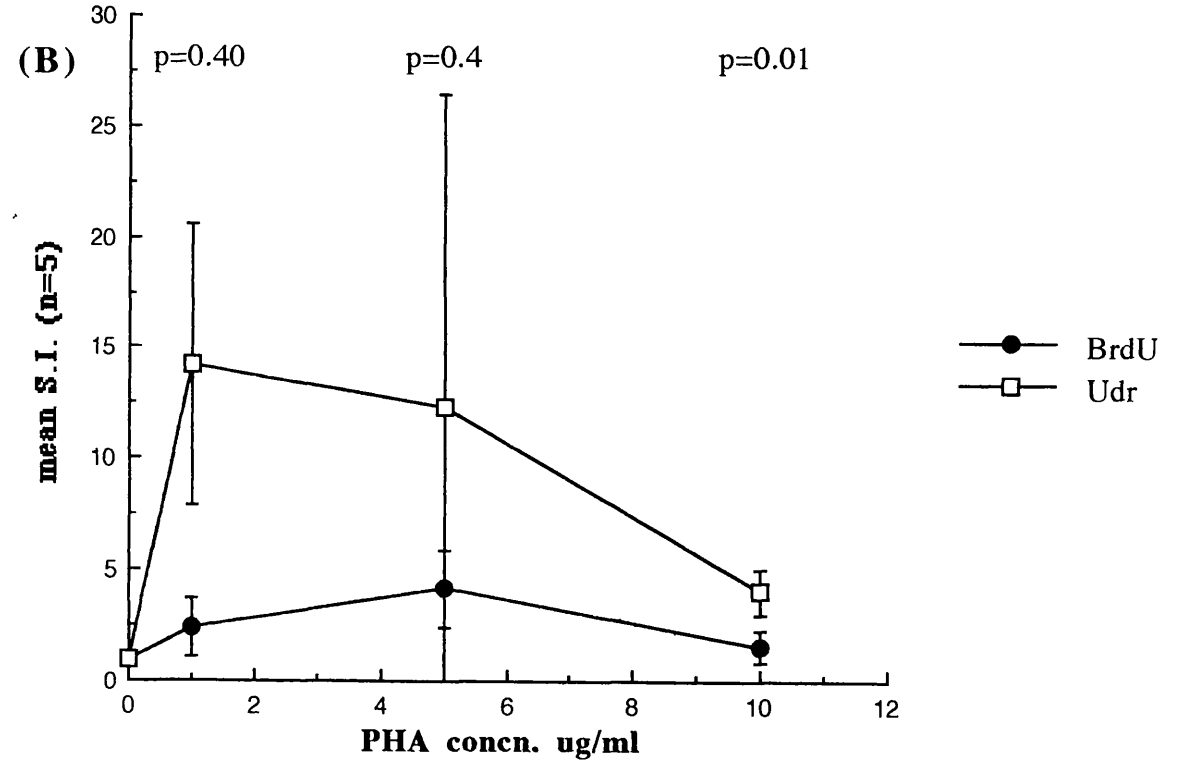


Figure 40. Graphical representation of the data comparing the incorporation of Udr and BrdU in PBLs from A) chronically infected (C) and B) uninfected (U) woodchucks after stimulation with PHA. PBLs were incubated in Clicks medium supplemented with 0.5% WCS and pulsed after 4 days. p Values < 0.05 were considered significant.

2.4.11. Optimal Conditions for Culturing Woodchuck PBLs

The optimal culture conditions are summarised in table 16 below.

VARIABLE	OPTIMUM
Dilution of Blood	1 in 3
Separation of Media	Ficoll-Paque
Number of cells/well	1×10^5
Duration of Incubation	4 days
Medium	Clicks EHAA medium
Supplement	woodchuck serum
% Supplement	0.5%
Radioactive label	[^{3}H]-uridine (sp. act. 27Ci/mmol)
Length of Pulse	6 hours
Other Factors	2×10^{-5} M 2-ME

Table 16. Optimal conditions for culturing woodchuck PBLs

There was no significant difference in the background level of incorporation of Udr between chronically infected and uninfected animals as summarised in table 17.

Woodchuck (Number)	Basal Incorporation (Mean cpm ± S.E.M.)
Chronically Infected (5)	31 ± 11
Uninfected (5)	33 ± 18
p Value	0.85

Table 17. Basal level of incorporation of Udr into unstimulated woodchuck PBLs from chronically infected and uninfected animals incubated in Clicks medium supplemented with 0.5% WCS and 2-ME for 4 days. Mann-Whitney U-test p values <0.05 were considered significant.

There is a significant difference between the S.I. values obtained using 1ug/ml PHA from chronically infected and uninfected animals, but none at the higher concentrations (figure 41). (Mean cpm data in Appendix II, tables 34 and 35)

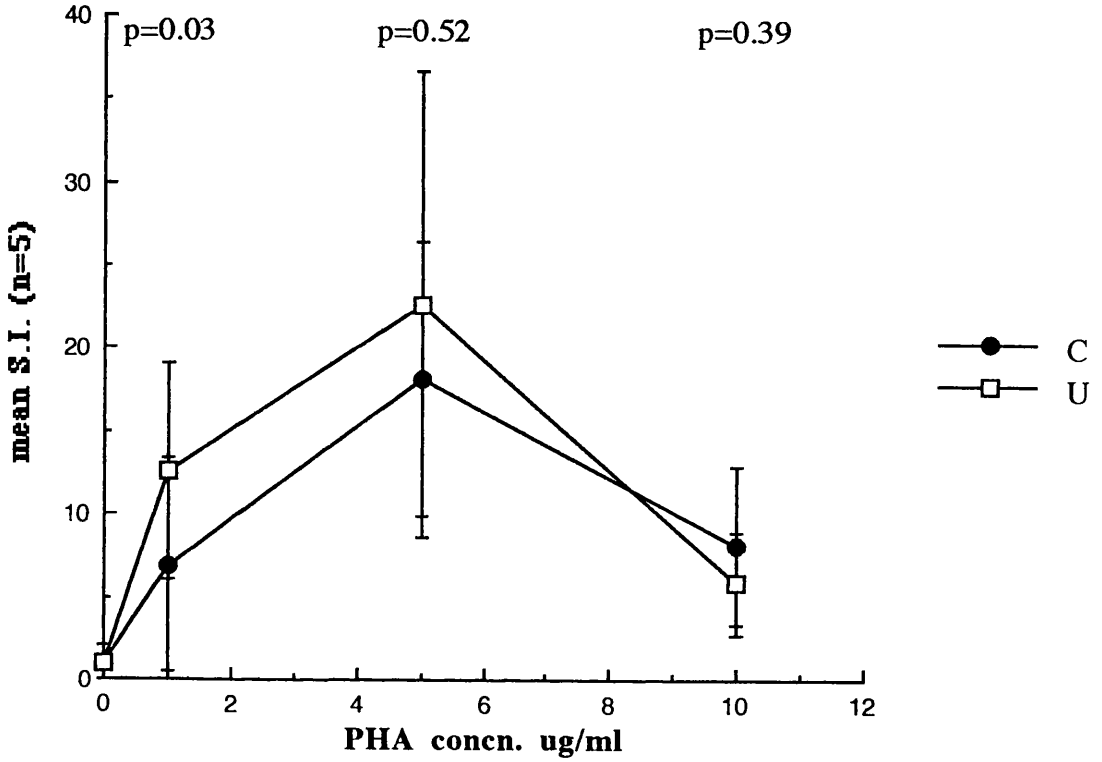


Figure 41. Graphical representation of the data comparing the response of PBLs from chronically infected (C) and uninfected (U) woodchucks after stimulation with PHA. PBLs were incubated under optimal conditions (table 16) and pulsed with Udr after 4 days. p Values < 0.05 were considered significant.

2.5 Discussion

The use of Ficoll-Paque yields lymphocytes of sufficient number and purity for use in cell culture assays from 4mls of woodchuck blood. It was found to perform better than both Percoll and metrizamide. Table 5 summarises the percentage composition of white blood cells before and after separation on Ficoll-Paque. The dilution of the blood 1 in 3 in medium reduces the number of lymphocytes which are trapped by aggregating red blood cells (RBC) so they do not sediment together and this produces an increased yield of lymphocytes.

Initial experiments involved the use of RPMI 1640 medium with 10% FCS as supplement as this has been used successfully to support T cell proliferation in the mouse and in human cultures. The background counts for assays in this group was found to be very high, in many cases higher than the test culture wells. In addition the S.I. values were extremely low indicating a low level of stimulation or a low level of incorporation of radioactive label. By changing the radioactive label to Udr S.I.s were marginally improved and Con A produced a significantly different S.I. at 1 and 10ug/ml (p value 0.01) in chronically infected animals. By trying different serum supplements it became clear that only mouse serum appeared to produce any significant change in the S.I.

The medium was changed and Clicks EHAA medium (Click et al 1972) substituted which, because of its high amino acid content (Appendix I) was able to support the

cultures when supplemented with only 0.5% serum. The use of serum supplements from other animal species in Clicks medium confirmed that only mouse serum appeared to have a significant effect on cell proliferation. Other groups have used the combination of 0.5% MS in Clicks medium to successfully culture mouse cells (Gao et al 1989, Zheng et al 1992).

Woodchuck serum can only be obtained by bleeding uninfected animals and in order to supplement the medium with 10% serum on a regular basis a large volume of blood was required. The use of Clicks medium, which only required supplementing with 0.5% serum, made it possible to regularly use WCS. Using Clicks medium with either 10 or 0.5% WCS resulted in a significantly higher S.I. than using MS in uninfected animals when cells were stimulated with PHA. In general WCS performed better than MS. WCS from uninfected animals was subsequently used as part of the final assay conditions; overall it produced more consistent and reproducible results.

Inclusion of 2-ME in the medium appears to favour woodchuck lymphocyte proliferation. No significant differences were observed but the S.I. values were higher in the presence of 2-ME. The reason for this is uncertain but speculation on its effect on glutathione levels exists. Reduced glutathione (GSH) can be oxidized to disulphide glutathione (GSSG) or to a mixed disulphide. Both are capable of scavenging free radicals which activates ornithine decarboxylase This is an early event involved in the cellular proliferation pathway. The maintenance of an intracellular glutathione level is therefore important for the protection of lymphocytes from free oxygen radicals. The thiol 2-ME is thought to enhance the availability of GSH which, through its radical

scavenging ability, prevents oxygen radical-induced cell membrane lipid peroxidation (Hoffeld 1981, Fidelus 1988). Alternatively, 2-ME could be reducing the mitogen and separating the molecules allowing greater access to lymphocytes which would increase the response.

A significantly higher response to PHA was observed at 1, 10 and 20ug/ml in uninfected animals although PHA generally performed better than the other mitogens in all animals. This observation is consistent with the response of human PBLs to PHA and Con A stimulation. Different populations of cells are stimulated by these two mitogens; Con A is more effective at stimluating CD8 cells whereas PHA is a potent stimulator of all T lymphocytes (Male et al 1991). LPS, which stimulates B cells in the mouse, was not found to be effective in stimulating woodchuck PBLs, contrary to observations by Korba et al (1988).

There was no significant difference between a 4 and 7 day incubation period although it was noted that a 4 day incubation period produced higher S.I.s than a 7 day one and in consequence was adopted as part of the optimal culture conditions.

The choice of radioactive label proved to be a large factor in defining the experimental parameters of a cell culture system for woodchuck lymphocytes. Thymidine (Tdr), with a variety of specific activities, was tried at a range of pulse times. Tdr uptake was detected but at a very low level. This could be because the cells do not respond to the mitogen by transformation and do not proliferate or Tdr inhibits the response and growth of these cells. Neither of these reasons appear to be the case as blast formation

and an increase in cell number in response to PHA was observed under the microscope. BrdU, a thymidine analogue, was incorporated in Clicks medium, albeit at a lower level than Udr. This indicates that DNA synthesis does occur and that the cells do respond to mitogen stimulation by transformation and that the kinetics of BrdUs uptake are the same as for Udr. The lack of incorporation of Tdr has been confirmed by Cote and Gerin (1995) who have successfully used uridine and adenosine to label the cells. Udr has also been used successfully by another group (Menne et al 1997) to measure stimulation of woodchuck lymphocytes.

Uridine incorporation was found to be very efficient in woodchuck PBLs with maximum incorporation occurring on day 4. The incorporation of BrdU indicates that the cells do enter the S phase of the cell cycle. The kinetics of Udr incorporation in response to various mitogen concentrations in human PBLs is comparable to Tdr incorporation, although it is approximately half that of Tdr.

Morphological blast transformation characterizes the progress from G0 to G1 of the cell cycle and also involves transcriptional activation of the IL-2 receptor and induction of Udr incorporation. During the cell cycle the activity of thymidine kinase increases indicating the proliferative state of the cell. However, woodchucks possess very low levels of thymidine kinase in their cells (Adelstein *et al* 1964) and therefore cannot make dTTP via the salvage pathway for incorporation into DNA. Instead it must utilise the *de novo* pathway which begins with the formation of UDP or CDP. Udr is incorporated directly into UDP which forms dUMP which acts as a precursor

for dTTP synthesis. The uptake of Udr reflects therefore the proliferation of the cells as measured by DNA synthesis.

Using sub-optimal conditions a variety of responses to mitogen were observed, although very few of these were of statistical significance. There was a significant difference between the response observed in chronically infected and uninfected animals with 1ug/ml PHA using the optimal conditions but not at the higher concentrations. The response appears to be lower in chronically infected animals compared to uninfected animals; this has also been observed in human patients chronically infected with HBV (Anastassakos et al 1987) where the addition of IL-1, IL-2, mixed lymphocyte reaction supernatant, monocytes or indomethacin failed to correct the impairment. This may suggest an impairment of T cell function in chronically infected animals which may contribute to the disease pathology.

In conclusion, maximum proliferation in response to mitogen was produced in cells cultured for 4 days using the culture conditions outlined in table 16. The introduction of these culture conditions reduced the amount of interassay variation. This system was used further to investigate the response to woodchuck core antigen peptides (chapter 3) and also to study the effect of various therapeutic strategies on the cellular immune response to various antigens (chapters 4 and 5).

Chapter 3

An Investigation of the Woodchuck T Cell Proliferative Response to Woodchuck Nucleocapsid Peptides

Published :

Shanmuganathan, S., Waters, J.A.., Karayiannis, P., Thursz, M., Thomas, H.C.

(1997) Journal of Medical Virology 52:128-135

3.1 Introduction

It is thought that in humans, HBcAg plays an important role in the initiation of the immune response by recruiting viral-specific T cells (CD4+) which are found at the site of liver cell injury. These CD4+ T cells then provide help to the cytolytic T cells (CD8+) which can lyze and eradicate infected liver cells. Previous work by Ferrari et al (1991) found that a difference in the Class II restricted T cell response exists between acute and chronic HBV patients to HBV nucleocapsid peptides which they suggest may be the cause of the different clinical outcomes of the disease. The lack of T helper cells may have a two pronged effect, in that the TH1 subset provides help to CTL to lyze infected hepatocytes and the TH2 subset provides direct assistance to B cells resulting in the production of virus-neutralizing antibodies. Whether this situation is similar in the woodchuck is unknown.

A T cell response may be detected in immunised animals in the absence of detectable anti-WHc (M. Roggendorf-personal communication) and therefore monitoring T cell responses after WHV inoculation is important. The following study aims to a) investigate the Th response to the nucleocapsid antigen in acute and chronic WHV infection, b) identify common or immunodominant T cell epitopes within the antigen which may prove useful in monitoring the response and in the development of treatments for chronic disease and c) investigate the potential for peptide T cell vaccines.

3.2 Materials

Serum Used to Induce an Acute Infection - Woodchuck serum was obtained from W933 (WHV DNA positive by Dot blot analysis, a chronically infected animal that developed a large liver carcinoma with a high GGT level of 1160 IU/L). 0.5ml was used to inoculate W30 subcutaneously (s.c.) and 0.5 ml was also used to inoculate W939 intravenously (i.v.). 1ml of serum (kindly provided by Dr. L.Cova) was later used to inoculate W939 s.c.

Animals - Experimental acute infection was attempted in two uninfected animals (for definition of uninfected animal see chapter 2, section 2.2). Table 20 summarises their status.

Woodchuck Number	WHV DNA	WHeAg	anti-WHc
W30	negative	negative	negative
W939	negative	negative	negative

Table 18. Summary of the status of the two uninfected woodchucks used for inoculation with infected sera.

WHV Core Antigen (WHcAg) - Attempts to express and purify the WHcAg did not result in sufficient quantities of protein for further work. As a result peptides were used which were made by Zeneca and designed to be 15 amino acids in length with 7-8 overlapping amino acids (table 19).

Peptide Number	Amino acid sequence
1. $Core_{1-15}$	M D I D P Y K E F G S S Y Q L
2. $Core_{8-22}$	E F G S S Y Q L L N F L P L D
3. $Core_{16-30}$	L N F L P L D F F P D L N A L
4. $Core_{23-37}$	F F P D L N A L V D T A T A L
5. $Core_{31-45}$	V D T A T A L Y E E E L T G R
6. $Core_{38-52}$	Y E E E L T G R E H C S P H H
7. $Core_{46-60}$	E H C S P H H T A I R Q A L V
8. $Core_{50-69}$	T A I R Q A L V C W D E L T K
9. $Core_{61-75}$	C W D E L T K L I A W T S E Q
10. $Core_{68-82}$	L I A W T S E Q V R T I I V N
11. $Core_{76-90}$	V R T I I V N H V N D T W G L
12. $Core_{83-97}$	H V N D T W G L K V R Q S L W
13. $Core_{91-105}$	K V R Q S L W F H L S C L T F
14. $Core_{98-112}$	F H L S C L T F G Q H T V Q E
15. $Core_{106-120}$	G Q H T V Q E F L V S F G V W
16. $Core_{113-127}$	F L V S F G V W I R T P A P Y
17. $Core_{121-135}$	I R T P A P Y R P P N A P I L
18. $Core_{128-142}$	R P P N A P I L S T L P E H T
19. $Core_{136-150}$	S T L P E H T G G A R A S R S
20. $Core_{143-157}$	G G A R A S R S P R R R T P S
21. $Core_{151-165}$	P R R R T P S P R R R R S Q S
22. $Core_{158-172}$	P R R R R S Q S P R R R S Q S
23. $Core_{166-178}$	P R R R S Q S P S A N C

Table 19. Synthetic Peptides Constructed from the WHV 8' Core Region

Woodchucks - Table 20 summarises all animals used in the T cell proliferation assays using peptides. All animals were obtained from an area endemic for WHV7 and WHV8 (New York State, USA).

Woodchuck	Chronically Infected (C), Uninfected (U) or Recovered (R)	WHV DNA (pg)	WHeAg(P.Erlich U)	GGT Levels (IU/L)
W859	U	0	0	<2.8
W32	U	0	0	<2.8
W867	U	0	0	<2.8
W939	U	0	0	<2.8
W507	C	103.21	75.20	<2.8
W518	C	50.18	62.47	<2.8
W520	C	11.30	68.82	10.90
W526	C	82.75	75.92	<2.8
W527	C	146.94	77.86	<2.8
W540	C	260.13	71.82	14.6
W556	C	54.01	75.45	4.94
W936	C	11065	583.04	424
W937	C	13800	658.56	68.7
W360	C	119.22	13.26	<2.8
W363	C	139.37	30.57	<2.8
W414	C	104.55	20.62	<2.8
W406	R	0	0	<2.8
W407	R	0	0	<2.8

Table 20. Summary of the Data Indicating the Status of All Animals Used in the Peptide Study

GGT- Reflotron kit (Boehringer Mannheim).

WHeAg - Enzymun Test for anti-HBe and HBeAg (Boehringer Mannheim).

anti-WHc - Enzymun Test for anti-HBc and HBcAg (Boehringer Mannheim)

3.3 Methods

3.3.1. Acute Infection

W939 was inoculated i.v. with 0.5ml of serum of unknown viral titre from W933. W30 was given 0.5ml of the same inoculum s.c. Two different routes were attempted as both have been reported to be successful in young animals. Both animals were monitored by weekly blood sampling and isolation of lymphocytes (including before inoculation) for testing with PHA and peptides. W939 was later administered 1ml of infectious serum of unknown viral titre, but known to result in acute infection of uninfected woodchucks (kindly provided by Dr. L. Cova). T cell responses to peptides were monitored as was the development of anti-WHc.

3.3.2. T Cell Proliferation Assays

The woodchuck was anaesthetized and 5mls of blood removed by venepuncture of the antecubital vein of the hind legs. Heparin was added (10U/ml) and the blood diluted 1 in 3 with RPMI medium before being layered onto 7.5mls Ficoll-Paque. The gradient was centrifuged in an MSE centrifuge 3000 for 30' at 1000rpm at 20°C and the layer of lymphocytes at the blood/media interface was removed. The cells were washed three times in RPMI medium at 1800, 1500 and 1200 rpm consecutively and resuspended in Clicks medium supplemented with 0.5% woodchuck serum, 2mM glutamine and 2 x 10^{-5} M 2-ME, which was filter sterilized. The cells were then resuspended at a concentration of 1 x 10^6/ml and plated out into 96-well microtitre plates. Each well contained 100ul (1 x 10^5) cells plus 100ul peptide or the PHA

mitogen. Peptides were made up in DMSO at 10mg/ml concentration and were tested at three concentrations in triplicate - 1, 5 and 10ug/ml with 5ug/ml PHA acting as a positive control for 4 and 7 days. The final concentration of DMSO in the well was less than 0.1%. 4 day incubations were found to produce optimal proliferation and this time scale was used in further experiments. Each test was carried out in triplicate with three control wells containing cells and complete medium only.

The culture plate was sealed in a plastic bag and incubated for four days (5% CO2, 37°C). During the last 6 hours of culture the cells were pulse labelled with 1uCi ^{3}H-uridine (specific activity 27Ci/mmol) and the cultures then harvested onto glass fibre filters. After the filters were air dried for approximately 2 hours they were counted on a Matrix 96 counter for 3 minutes. Proliferation was expressed as a stimulation index (S.I.) calculated as the mean cpm of triplicate values in the presence of 5ug/ml peptide /mean cpm in the absence of peptide.

Statistical Evaluation of Positive S.I.

S.I.s of greater than 3.5 were considered significant indicators of proliferation. This figure was calculated from the mean of the S.I.s obtained in response to 5ug/ml of all peptides by the uninfected animals ± 2 standard deviations (s.d.) from the mean.

In order to obtain spleen cells an animal was sacrificed by exsanguination. The spleen was removed and placed in 10mls of medium. The organ was teased apart using sterile needles and the liberated cells washed once in RPMI medium, counted and

resuspended in Clicks complete medium (chapter 2) at 1 x 10^6cells/ml. Frozen cells were found to perform as well as freshly obtained cells.

3.4 Results

3.4.1. Results of Acute Infection

W30 was 2kg in weight and was slightly emaciated in appearance prior to inoculation. It developed an acute infection but did not yield a large amount of blood and consequently it was difficult to obtain T cell responses to peptides. The animal became more emaciated and had difficulties breathing unrelated to the hepatitis. The animal was sacrificed 7 weeks after inoculation and 3 weeks after development of an acute infection. Table 21 summarises the changes in the parameters for W30. The animal could not be followed through the whole period of infection and the pattern of the T cell response to peptides during acute infection could not be determined.

Time/week	WHeAg/U	WHV DNA/pg	anti-WHc	HBc S.I.	PHA S.I.
1	0	0	-ve	n.d.	n.d.
2	0	0	-ve	n.d.	n.d.
3	0	0	-ve	1.46	3.70
4	0	0	-ve	2.04	5.89
5	242.16	26.06	+ve	n.d.	n.d.
6.	588.16	344.26	+ve	n.d.	n.d.
7.	566.52	303.77	+ve	12.21	103.61

Table 21. Parameters monitored for the Development of Acute Infection in W30. GGT Levels remained <2.8IU/L. (-ve - negative, +ve - positive)

W939 remained WHV DNA negative, WHeAg negative and anti-WHc negative following each inoculum. Table 22 outlines the T cell response to peptides 5 and 8 weeks after i.v. administration of inoculum. There is a large increase in the response to peptide 98-112 in particular. There is also a doubling of the T cell S.I. to petides 8-22, 16-30, 50-69, 83-97, 91-105 and 106-120.

3.4.2. Chronic Carrier Status of the Woodchucks

The WHV status of the chronically infected animals shows that all who were WHV DNA positive were also WHeAg positive (table 20). Laboratory infected animals were infected at birth and at the time of testing were 8 months old. The length of time wild caught woodchucks have harboured the virus is unknown and therefore the effect of this on the T cell response to peptides cannot be determined. The measurement of GGT levels was used to monitor the development of HCC. Only two of the chronically infected animals, W936 and W937, showed evidence of highly elevated levels of GGT indicating development and advancement of HCC. Their responses to peptides however, were markedly different (table 24). Most of the chronically infected animals did not have HCC at the time of testing for peptide responses as determined by their GGT levels.

Peptide	T cell S.I.	
	Week 5 After Inoculation	Week 8 After Inoculation
1. $Core_{1-15}$	3.87	3.24
2. $Core_{8-22}$	2.98	6.02 *
3. $Core_{16-30}$	3.10	6.05 *
4. $Core_{23-37}$	3.51	4.56
5. $Core_{31-45}$	3.21	5.24
6. $Core_{38-52}$	3.10	2.82
7. $Core_{46-60}$	3.02	4.23
8. $Core_{50-69}$	3.00	7.05 *
9. $Core_{61-75}$	5.61	3.48
10. $Core_{68-82}$	3.60	5.30
11. $Core_{76-90}$	4.98	3.93
12. $Core_{83-97}$	4.00	8.60 *
13. $Core_{91-105}$	4.83	9.17 *
14. $Core_{98-112}$	2.74	13.41 *
15. $Core_{106-120}$	3.02	6.72 *
16. $Core_{113-127}$	5.25	7.79
17. $Core_{121-135}$	3.76	1.65
18. $Core_{128-142}$	2.86	2.44
19. $Core_{136-150}$	2.38	1.53
20. $Core_{143-157}$	3.25	1.65
21. $Core_{151-165}$	5.48	1.72
22. $Core_{158-172}$	4.67	2.84
23. $Core_{166-178}$	4.89	4.27

Table 22. T cell responses of PBLs from W939 (WHV DNA negative, WHeAg negative, anti-WHc negative) WHcAg peptides 5 and 8 weeks after i.v. inoculation with 0.5ml of infectious serum of unknown viral titre. The animal did not develop viraemia and was therefore protected (the same inoculum produced infection in W30).

3.4.3. Dose Response to Peptides

PHA was found to produce a maximum T cell response after 4 days at 5ug/ml; this was also true of peptides (figures 42-44). Each peptide was tested at 3 concentrations - 1, 5 and 10ug/ml and 5ug/ml was found to be the optimal concentration for each peptide in each animal. Examples of this are illustrated in figures 42-44 using 2 uninfected animals, 1 naturally chronically infected and 1 laboratory chronically infected animal. The response time of woodchuck PBLs to both mitogen and peptides appears to be the same. This is unlike the situation in humans where the response time to peptides is longer than to PHA.

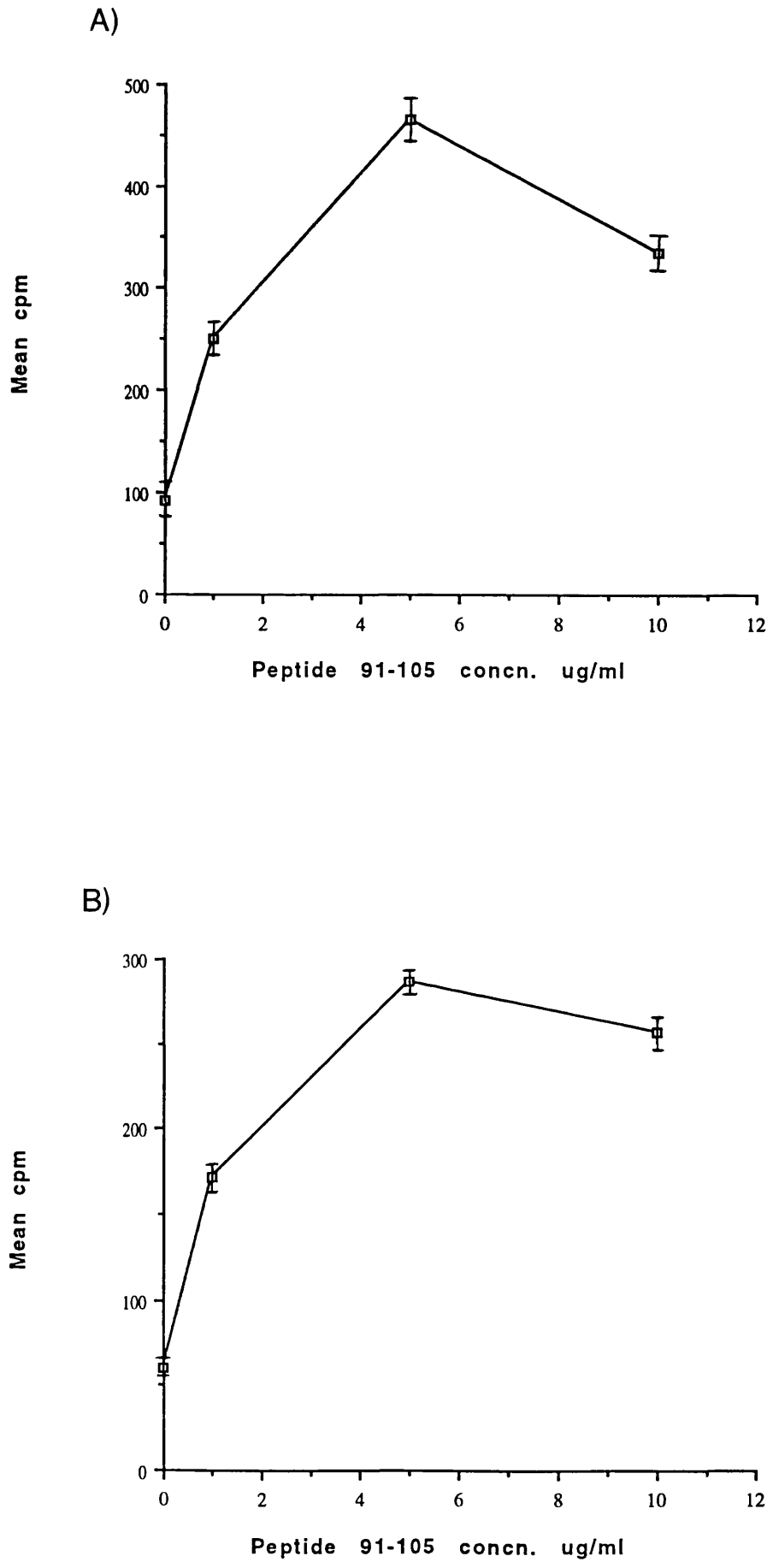


Figure 42. Graphical illustration of the dose response of T cells obtained from 2 uninfected animals in A) W30 and B) W867 to peptide 91-105. T cell responses are represented as mean cpm ± s.e.m.

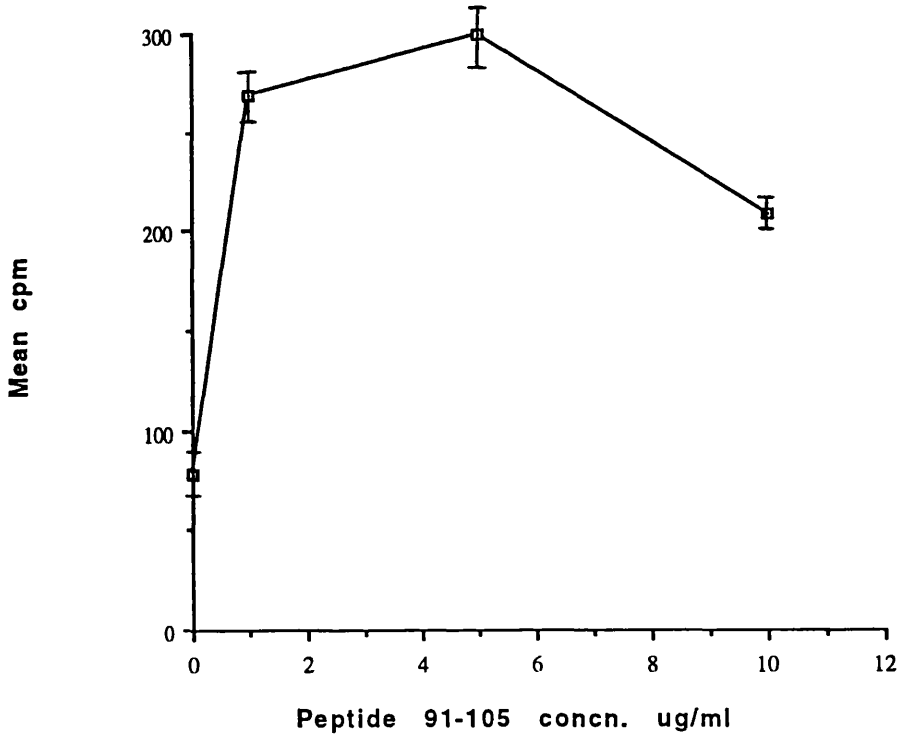


Figure 43. Graphical illustration of the dose response of T cells obtained from a laboratory infected chronic animal (W414) to peptide 91-105. T cell responses are represented as mean cpm ± s.e.m.

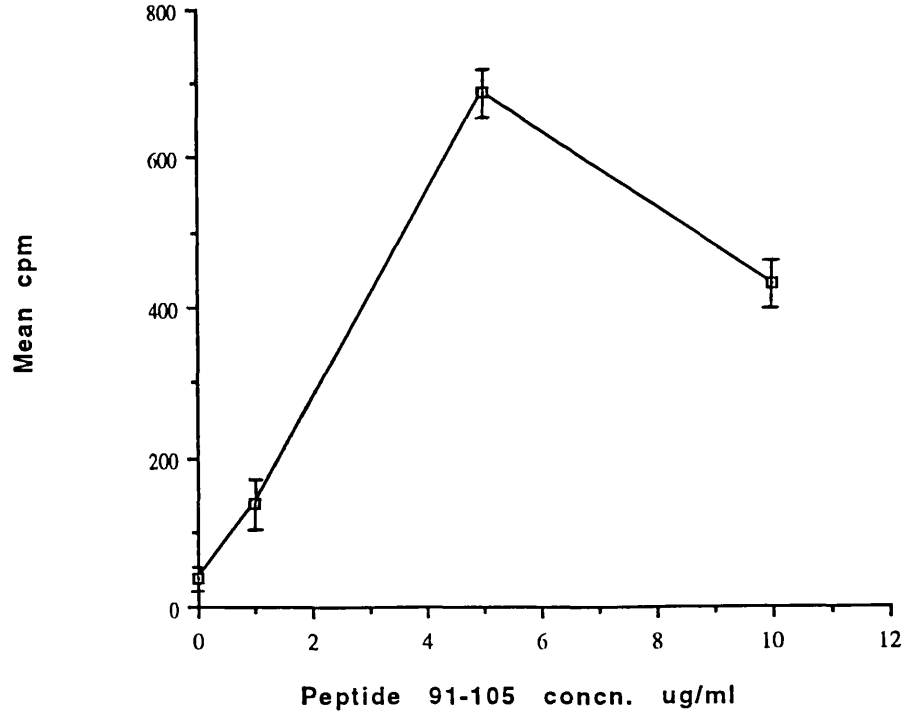


Figure 44. Graphical illustration of the dose response of T cells obtained from a naturally infected chronic animal (W518) to peptide 91-105. T cell responses are represented as mean cpm ± s.e.m.

3.4.4. Response to Peptides

The background cpm in the absence of peptide are summarised in table 23. All chronic carriers responded to at least one peptide with a maximum of 8 out of 12 animals responding to peptides 16-30, 38-52, 50-69, 76-90 and 91-105 (table 24). The number of animals who responded to each peptide is outlined in table 25. W937, infected in the wild, responded to all peptides and W540, also infected in the wild, responded to all except 2 peptides. The maximum T cell S.I. to be found in the greatest number of animals was directed against peptide 91-105. 5 out of 12 chronic carrier animals responded maximally to this peptide and only one was laboratory infected (W414).

In addition those animals who had a transient infection and then recovered (W406 and W407) both responded to peptide 31-45. They were tested approximately 6 months after they had recovered from their infection. In addition 5 of the naturally infected and 1 of the laboratory infected chronic animals also responded to this peptide. W556 responded to this peptide with a maximum T cell S.I. of 16.09. There was no response from any of the uninfected animals. In the uninfected animals few peptides produced an S.I. greater than 3.5 and where this did occur S.I.s were no higher than 5.

Woodchuck	Chronic (C), Uninfected (U) or Recovered (R)	Mean cpm from UnstimulatedCultures ± S.E.M.
W32	U	103.6 ± 18.9
W867	U	59.6 ± 15.1
W859	U	89.3 ± 20.6
W939	U	73.6 ± 17.5
W507	C	23.5 ± 1.7
W518	C	39.4 ± 4.2
W520	C	20.3 ± 3.7
W526	C	34.7 ± 2.0
W527	C	36.7 ± 4.1
W540	C	32.1 ± 8.0
W556	C	50.4 ± 10.6
W936	C	30.7 ± 1.4
W937	C	100.6 ± 20.8
W360	C	46.9 ± 7.2
W363	C	45.2 ± 7.6
W414	C	78.4 ± 2.3
W406	R	22.5 ± 3.9
W407	R	29.6 ± 2.9

Table 23. Basal level of incorporation of [^{3}H]-uridine in unstimulated PBL cultures. T cell responses are represented as mean cpm of triplicate values (± S.E.M.) and were used to calculate the S.I. values in table 24.

Woodchucks- Chronic WHV Infection

Peptide	Uninfected Animals				Naturally Infected									Laboratory Infected			Recovered	
	859	32	867	939	507	518	520	526	527	540	556	936	937	360	363	414	406	407
1. 1-15	1.29	1.63	0.69	2.52	4.43	1.31	1.55	0.95	0.98	2.99	1.44	2.03	25.61	2.14	2.19	0.76	1.48	0.43
2. 8-22	1.49	1.70	3.02	2.15	3.96	5.55	0.58	1.64	1.46	8.30	4.64	1.86	19.60	0.53	1.61	1.01	1.36	0.46
3. 16-30	4.27	2.18	1.54	2.21	6.71	2.92	4.57	0.89	2.84	22.79	10.64	7.39	37.29	5.14	3.72	1.42	1.10	2.65
4. 23-37	3.91	3.19	0.91	1.58	4.26	1.71	4.19	1.48	2.14	9.39	13.91	4.13	34.36	7.09	2.80	1.65	0.65	1.82
5. 31-45	3.31	2.80	1.32	1.28	1.92	1.46	4.94	1.27	2.30	13.90	16.09	4.14	29.76	2.43	4.51	1.41	6.44	11.08
6. 38-52	2.37	1.21	1.21	1.86	2.10	4.15	11.85	3.25	6.92	17.37	15.15	4.07	33.51	2.30	2.14	0.56	0.94	1.57
7. 46-60	1.16	1.61	0.87	2.52	16.17	2.32	4.08	1.19	2.16	3.61	12.00	4.91	38.34	2.29	1.71	0.73	0.88	1.73
8. 50-69	2.55	2.17	0.91	1.47	5.47	2.11	3.88	0.97	2.00	9.53	3.50	6.28	51.23	3.83	3.62	1.22	1.71	0.73
9. 61-75	1.67	2.23	0.99	1.28	4.75	1.59	4.94	1.18	0.93	2.60	0.61	5.84	51.59	1.51	1.97	0.72	0.88	2.15
10. 68-82	1.95	1.70	1.33	2.18	8.17	2.59	6.17	1.08	0.99	10.03	3.44	3.21	35.08	2.14	1.77	0.73	1.84	0.92
11. 76-90	1.49	1.78	1.35	2.06	6.05	2.09	10.64	2.33	1.00	14.65	3.45	19.74	46.76	3.49	1.18	3.46	2.39	0.70
12. 83-97	1.29	1.67	3.60	1.37	1.87	2.51	4.01	1.76	0.53	7.98	1.86	2.08	34.15	3.10	0.57	2.24	1.20	0.73
13. 91-105	3.03	2.47	4.82	2.43	24.30	17.42	3.86	4.61	0.89	17.05	1.84	35.99	66.45	1.83	1.43	3.82	1.27	0.74

Peptide	859	32	867	939	507	518	520	526	527	540	556	936	937	360	363	414	406	407
14. 98-112	2.06	2.40	3.20	1.90	5.06	2.28	3.84	1.14	0.98	8.76	2.51	2.79	21.91	2.75	1.21	1.68	1.27	1.10
15. 106-120	1.64	1.75	2.89	0.98	4.59	2.33	4.84	1.05	1.47	9.57	2.13	2.62	25.58	1.94	0.90	1.51	1.73	0.87
16. 113-127	2.13	1.53	3.03	2.00	4.79	1.80	3.60	0.99	1.08	30.37	1.80	4.50	37.71	1.62	0.57	2.51	1.88	0.52
17. 121-135	1.67	1.26	2.85	1.44	1.44	12.51	6.02	0.93	1.01	15.14	2.62	2.85	29.73	0.87	0.88	2.44	1.57	0.90
18. 128-142	1.41	0.80	4.04	0.76	1.76	2.89	3.96	1.10	0.67	11.37	2.37	2.33	25.97	0.84	0.45	2.30	1.56	0.66
19. 136-150	1.69	1.46	3.87	0.91	4.88	2.13	3.61	0.72	0.53	11.36	2.61	2.50	26.37	0.80	0.71	2.35	1.85	0.57
20. 143-157	2.45	1.34	2.73	1.00	8.23	17.42	6.56	5.19	1.28	34.91	2.92	3.94	37.86	0.59	1.96	2.44	1.28	0.87
21. 151-165	2.75	1.88	3.02	2.72	7.42	4.00	0.79	2.89	1.00	9.45	7.02	2.31	25.48	0.99	1.01	0.53	1.86	1.00
22. 158-172	2.57	2.75	2.81	2.11	6.63	4.04	0.76	2.41	1.32	13.88	6.06	2.31	21.31	0.65	2.09	0.82	1.19	0.49
23. 166-178	1.63	2.03	1.81	2.43	1.48	5.16	0.53	1.07	2.25	15.27	12.98	2.52	28.50	0.75	1.40	0.66	1.08	0.66

Table 24. Summary of T cell Responses to Woodchuck Nucleocapsid Antigen Peptides in Uninfected (Naive), Chronically Infected and Recovered Animals. Results are expressed as a Stimulation Index (S.I.) at the optimum concentration of 5ug/ml. The cutoff S.I. was determined as 3.5 (mean of S.I.s obtained from uninfected animals ±2 s.d.). W939 was tested before infectious sera was administered.

Peptide	No. of Woodchucks that Responded with an S.I.>3.5	No. of Woodchucks with Maximum T cell Responses
1. $Core_{1-15}$	2	0
2. $Core_{8-22}$	5	0
3. $Core_{16-30}$	8	0
4. $Core_{23-37}$	7	1
5. $Core_{31-45}$	6	1
6. $Core_{38-52}$	8	2
7. $Core_{46-60}$	6	0
8. $Core_{53-67}$	8	0
9. $Core_{61-75}$	4	0
10. $Core_{68-82}$	6	0
11. $Core_{76-90}$	8	0
12. $Core_{83-97}$	4	0
13. $Core_{91-105}$	8	5
14. $Core_{98-112}$	4	0
15. $Core_{106-120}$	4	0
16. $Core_{113-127}$	5	0
17. $Core_{121-135}$	4	0
18. $Core_{128-142}$	3	0
19. $Core_{136-150}$	4	0
20. $Core_{143-157}$	7	2
21. $Core_{151-165}$	5	0
22. $Core_{158-172}$	5	0
23. $Core_{166-178}$	4	0

Table 25. Summary of the total number of chronically infected woodchucks who responded to each peptide and the number of chronically infected woodchucks in whom a peptide produced the highest T cell response.

3.5 Discussion

In humans the HBV nucleocapsid antigens induce a T cell mediated response and are targets for immune-mediated attack (Milich et al 1986, Mondelli et al 1982). The ability of both HBcAg and WHcAg to stimulate a protective immune response against HBV and WHV infection, in chimpanzees and woodchucks respectively (Iwarson et al 1985, Roos et al 1989), indicates the importance of these antigens during the course of infection.

The results of this study show that all 12 chronically infected animals, whether infected in the wild or in the laboratory, responded to at least one nucleocapsid peptide and a maximum of all 23 peptides. Of the 5 animals that responded to fewer than 5 peptides 3 were laboratory infected. In this group 8 months had elapsed since infection whereas the length of time for which the wild caught animals had been infected was unknown. Both W936 and W937 had high GGT levels which indicated HCC development and suggested that they had harboured the virus for a period of 2-3 years (Popper et al 1987). Both of these animals had significant T cell responses to a large number of peptides; W936 responded to 12 peptides and W937 to all 23 peptides. These results may indicate that the longer the animal has harboured the virus, the greater the T cell response to the nucleocapsid antigens. Although W507 had no indication of tumour development at the time of testing it responded to a broad spectrum of peptides. W520, whose GGT level was 10.90, did not develop a tumour after testing (GGT levels above 10IU/L indicate tumour) and also responded to a range of peptides. The length of time both animals were infected with WHV

could not be assessed. Although they had not developed HCC at the time of testing they may have been harbouring the virus for some time.

The remaining chronically infected animals mounted a significant T cell response to a minimum of 8 peptides of which all, except W556, responded to peptide 91-105. Indeed in five chronically infected animals a maximum S.I. was induced in response to this peptide. Together these results suggest that this sequence of 15 amino acids is an important T cell epitope in woodchucks. It does not coincide precisely with epitopes reported to be important in humans, although it does partly overlap peptide 81-105 which was found to be immunogenic in 37% of acute and chronic patients who responded to core antigen (Jung et al 1995).

In humans two groups have identified T cell epitopes in the acute disease which are dominant irrespective of the HLA haplotype. Ferrari et al (1991) showed that 90% of patients with acute HBV responded to a peptide of amino acids 50-69. Jung et al (1995) have identified peptides 1-25 (52% responded) and 61-85 (59% responded). Although nothing is yet known about the MHC of the woodchuck, these animals are an outbred population and it seems likely that peptide 91-105 represents an epitope to which T cells respond independent of their HLA haplotype. The difference between the core nucleotide sequences of WHV 7 and WHV 8 is 0.5% (Cohen et al 1988) which may account for differences in the response.

Although during acute disease there is a marked T cell response to nucleocapsid antigens, 45% of patients responded, this is much less frequent in chronic carriers

where 13% of patients responded (Jung et al 1995). In acute exacerbations of chronic disease and during HBeAg/HBe antibody seroconversion an increased T cell responsiveness is observed. In comparison, the 66% of responders in the group of chronic carrier woodchucks studied is much higher than the response seen in chronic carrier HBV patients. It has been suggested that because chronically infected woodchucks demonstrate liver core antigen and persistent circulating DNA polymerase activity, the disease is akin to the more aggressive form of chronic liver disease in the human (Ponzetto et al 1984). If this is the case we would expect a more vigorous T cell response in chronic carrier woodchucks, like the ones observed in this study, than in chronic carrier patients.

It is interesting to note that in those animals who recovered from infection (W406 and W407) a response to peptide 31-45 was observed. This indicates that nucleocapsid specific T cells are still circulating in the periphery. However, whether they were responsible for clearance of the infection cannot be determined. This was the only peptide to which these animals responded although some chronic carriers also had a response to this peptide. It is possible that these two animals responded to this peptide because they possess the MHC restriction element necessary to present this peptide. Also more than one MHC Class II molecule may be able to present each peptide. This peptide may be worthy of further investigation using more animals who have recovered from infection.

Despite several attempts to inoculate uninfected animals only one developed virologically detectable acute infection (W30). Other groups have produced acute

infections in newborn or very young woodchucks who appear to be more susceptible to infection (Tyler et al 1986, Popper et al 1987, Korba et al 1989,1990) and this may be one of the reasons why no infection resulted in some of our adult animals. W939 resisted 3 attempts to be infected with different inocula by different routes of administration. It is of interest to note that the T cell responses to peptides after inoculation did not appear until 8 weeks after which time the animal was anti-WHc negative.

A recent study be Menne et al (1997) suggests that T cell responses are crucial for protection from WHV infection, more so than antibody production. They found that 8 acutely infected woodchucks responded strongly to one WHcAg peptide covering the region 97-110. Furthermore they found that when animals were immunised with this peptide they were found to be protected from infection upon challenge but did not produce any anti-WHc. They did however, have increased T cell proliferative responses. Those animals immunised with recombinant WHcAg were also protected but developed high anti-WHc levels. This study shows that it is possible to remain immune to WHV infection without the development of anti-WHc. W939 has no detectable levels of anti-WHc but does have an increased T cell proliferative response to a similar peptide (region 98-112) found to be immunodominant in acutely infected woodchucks. W939 may be immunised after WHV infection and remains so because of a strong T cell response rather than an anti-WHc response. In rare cases of HBV infection in humans no anti-HBc can be detected but the patients do not develop infection. W939 did not develop any serological markers of infection in 10 weeks of follow up.

Further work in this area could compare T cell responses between acutely and chronically infected animals. In addition, by following them over a period of time variation in responses can be observed and compared to responses to HBV in humans.

Chapter 4

An Investigation of the Effect of Chemical Immunomodulators on Persistent WHV Infection

4.1 Introduction

Immune related mechanisms, such as tolerance and anergy, have a major impact on disease progression to chronicity, as seen in the HBeAg transgenic mouse model (Milich et al 1990). Th cells may play an important role in chronic disease and in order to eradicate virus a means of up-regulating these cells or increasing their number may be essential. However, information concerning the role of Th cells in chronic hepatitis B infection is limited due to the lack of a suitable animal model in which to study the immunopathogenesis of infection. The woodchuck now fills this void and, having established parameters which can be used to assess the effects of treatments, has allowed us to carry out trials of an immunopotentiating drug with the generic name Tucaresol.

Tucaresol acts at the T cell level by enhancing the activation signal between surface ligands on antigen presenting cells (APCs) and T cells by increasing Schiff base formation. Administration of this compound, either as a drug treatment on its own or as an adjuvant with a nucleocapsid antigen, may have an effect on the progression of disease. A dose related study is undertaken using chronically infected animals in order to ascertain whether the drug can induce a clinically relevant response, in this case the clearance of WHV DNA from the serum, and whether the drug has an anti-tumour effect.

In addition a drug with the name MPC-866, is used on woodchucks with developing HCC in an attempt to arrest tumour development. MPC-866 is a broad spectrum anti-

viral drug which has been found to have anti-tumour effects; it acts by binding to the primer-template groove of the reverse transcriptase enzyme. This molecule has been suggested as an alternative to AZT in HIV patients but as yet has not undergone any trials. This trial provides the opportunity to test the potential of this drug in an animal model of a viral disease. The following preliminary studies will seek to outline a chemical approach to immunomodulation in the WHV chronically infected woodchuck, and the outcome will be used to assess its potential for augmenting the immune response.

4.2 Materials

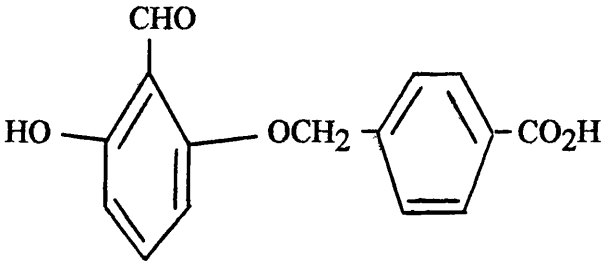
6 chronic carrier woodchucks naturally infected in the wild : W933, W935, W936, W937, W507, W518. 4 chronic carriers laboratory bred and infected : W360, W363, W405, W414. Maintained as indicated in chapter 2.

WHV Dot blot : All radiochemicals were purchased from Amersham, all chemicals from BDH, SIGMA, GIBCO BRL or Boehringer Mannheim and medical X-ray film from Fuji. Recombinant HBcAg was obtained from the Wellcome Foundation.

Tucaresol - manufactured by Glaxo/Wellcome. Known chemical properties outlined in table 26.

TUCARESOL : -

Structural Formula :



Generic Name	Tucaresol
Chemical Name	4-[(2-formyl-3-hydroxyphenoxy)methyl] benzoic acid
Molecular Formula	$C_{15}H_{12}O_5$
Molecular Weight	272.26
Chemical Properties	insoluble in water, sparingly soluble in 0.1M NaOH, melting point 242°C.
Shelf Life	5 years at 30°C
Mode of Action	Schiff base formation

Table 26. Chemical Properties of Tucaresol

MPC-866:-

MPC-866 is a member of a new class of synthetic oligonucleotides which act as broad spectrum anti-viral drugs. A high degree of potency and a low level of toxicity have been observed when used in cell culture systems against HIV and HCMV. It consists of 32 monomer units with a phosphodiester backbone and a 3'-hydroxyhexyl phosphate ester tail. It forms a stable duplex in solution and its action is non-sequence specific. Its predecessor (MPC-531) was designed to inhibit the activity of HIV-1 reverse transcriptase by binding to the primer-template groove of the enzyme. MPC-866 is thought to operate in a similar fashion by binding to a DNA duplex and preventing its transcription.

4.3 Methods

Study 1. Tucaresol

DRUGS AND DOSAGE :

Preparation of 10mg/ml solution : 330mg of Tucaresol was weighed out and 33mls of PBS added. 10M NaOH was added dropwise until the powder dissolved to form a yellow solution (pH 9.5-10). The pH was returned to ~8.5 by the addition of concentrated HCl dropwise. Preparation of 120 and 80mg/ml solutions : Same as above except 120mg was dissolved in 1ml PBS only.

Preparation of HBeAg : HBcAg (Wellcome Foundation) was denatured using 0.1% SDS and 0.1% 2-mercaptoethanol with incubation for 2 hours at 37°C. 250ug HBeAg was administered by intramuscular (i.m.) injection into the hind leg in addition to tucaresol to those animals indicated in table 27. The rationale behind this strategy is an attempt to break tolerance by administering an antigen that differs in amino acid sequence from the native WHeAg.

Animal Selection : the woodchucks used in these experiments are summarised in table 27.

Tucaresol Treatment

Duration of Dose : 14 days ; 7 doses on alternate days

Route of Administration : Intramuscular

Serum samples were taken every week, 3 weeks before the start of the first treatment and then as follows:-

W360 and W414 control animals -

Weeks	1-3	baseline pre-bleeds
Weeks	4-5	on treatment
Weeks	6-11	follow-up

W933, W937, W935, W936 given 20mg/kg -

Weeks	0-2	baseline pre-bleeds
Weeks	3-4	on first treatment
Weeks	5-12	follow-up of first treatment
Weeks	12.5-14	on second treatment
Weeks	15-22	follow-up of second treatment

Time course for W406, W407, W520, W527 given 30mg/kg

Weeks	1-3	baseline
Weeks	4-5	treatment

Weeks 6-10 follow-up

Time course for W507, W518, W360, W414, W363, W405 given 40mg/kg :-

Weeks 1-3 baseline pre-bleeds

Weeks 4-5 on treatment

Weeks 6-11 follow-up

Animal	Naturally (N) or Laboratory (L) Infected, Uninfected (U)	Weight / kg	Drug Volume /mls	Dose mg/kg	HBeAg
W933	N	5	10	20	-
W937	N	4	8	20	-
W935	N	3.5	7	20	+
W936	N	3.75	7.5	20	+
W406	U	4	1	30	+
W407	U	4	1	30	+
W527	L	2	2	30	-
W520	L	2	2	30	-
W360	L	2	2	40	+
W414	L	2	2	40	+
W507	N	2	2	40	-
W518	N	2	2	40	-
W363	L	3	1	40	+
W405	L	2	1	40	+
W360	L	2	C	C	
W414	L	2	C	C	

Table 27. The weight, WHV Status and the Dose of Tucaresol Used in Each Animal. In addition 4 other untreated naturally chronically infected animals (W1, W5, W10 and W16) were used as part of the control group for statistical analysis. Their WHV DNA levels were measured at two time points 6 weeks apart.

WHV DNA Levels by Dot Blot Analysis

100ul of woodchuck serum was digested with 20ul pronase (100mg/ml), 1.7ul 25% SDS, 2.5ul t-RNA (0.8mg/ml) and 75.8ul 2X Summers solution (Appendix I) for 16 hours at 37°C. Samples were extracted with an equal volume of phenol equilibrated with 1X Summers solution. After vortexing samples were centrifuged for 3' at 10,000xg and the aqueous layer removed. A 50ul aliquot was denatured by adding 100ul of 0.15M NaOH/1.5M NaCl and then vortexed for a few seconds. The solution was neutralised by adding 100ul of 0.15M Tris-HCl pH 7/1.5M NaCl after 5' and again vortexed. The samples were spotted onto a nitrocellulose filter which had been pre-wetted in distilled water and the filter was then baked at 80°C for 2 hours.

Probe Preparation : 120ng of linearized WHV DNA (obtained from purified plasmid DNA from E.coli pBR328) was labelled with γ-^{32}P-dCTP using the Nick Translation kit. Any unlabelled probe was separated by centrifugation on a G50 Sephadex column equilibrated in TE over siliconized glass wool at 1500rpm for 3'. The labelled probe was collected from the bottom of the tube and counted in a beta counter. The specific activity of the probe was usually between 4-8 x 10^8 cpm/ug DNA.

Dilutions of the linearised DNA were made ranging from 400 to 5pg in 50ul of sterile water. Samples were denatured, neutralised and spotted onto the filter as before, prior to incubation with the probe.

The filter was immersed in pre-hybridization solution (Appendix I) until saturated. The probe was boiled in 0.5ml hybridization solution (Appendix I) for 3' and applied to the filter with the remaining 4.5mls of hybridization solution and incubated at 37°C for 16 hours.

The filter was washed twice with solution 1 (5x SSC (Appendix I), 0.1% SDS, 50% formamide) at 37°C and twice with solution 2 (2x SSC, 0.1% SDS) at 65°C. The filter was air dried and autoradiographed with a pre-flashed film, using a flash gun, for 24 hours or longer at -70°C. Once the film had been developed the optical density of each spot was measured using a densitometer and the WHV DNA levels expressed as an integral of the area under the curve. Amounts of DNA were calculated using the standards. All samples from 2 animals undergoing the same treatment were processed in the same assay. Interassay variation which was estimated as 20% prior to running the samples.

WHeAg Antigen Levels

The WHeAg antigen levels were measured using the Enzymun test for anti-HBe and HBeAg (Boehringer Mannheim). A standard curve for each assay was carried out by diluting the positive control which contained a known number of units. All samples from 2 animals undergoing the same treatment were diluted 1 in 4 and processed in the same assay. The optical density (O.D.) for each was measured at 420nm. Results were expressed as Paul Erlich Units.

PBL Proliferation Assays

Assays were carried out as outlined in the methods section in chapter 3.5ug/ml PHA and HBcAg were used to monitor T cell responses. Proliferation was expressed as a Stimulation Index (S.I.).

Gamma Glutamyl Transferase (GGT) Levels

GGT levels were measured using a standard commercial assay kit from Boehringer Mannheim. Levels were expressed as International Units/Litre.

Statistical Analysis

The presence or absence of WHV DNA in the serum was assessed in chronically infected woodchucks who were untreated (n=6), treated with tucaresol only (n=6) and those treated with tucaresol and HBeAg (n=8) at a fixed time interval of 6 weeks. This is the time point at which WHV DNA is thought to be cleared. The difference in the WHV DNA levels of the 3 groups was then compared using the Mann-Whitney U-test to ascertain whether any differences between the groups was significant. A p value of <0.05 was considered significant.

Study 2. MPC-866

Drug preparation: the drug was made up in PBS at a concentration of 20mg/ml. The correct dosage was taken (1mg/kg) and diluted to 1ml in PBS. The drug was stored at 4°C. The woodchucks, W935 and W936, were chosen because of their elevated GGT levels. They were administered the drug i.m. daily for 11 days at a dose of 1mg/kg. Their GGT levels were monitored by the method outlined for the tucaresol study for a period of 30 days.

4.4 Results

Study 1. Tucaresol

4.4.1 Monitoring of Untreated Chronically Infected Animals (Controls)

A large degree of variation was seen for three parameters for the control animals (figures 45-48). There was no change in the GGT levels which remained <2.8IU/L. W1, W5, W10 and W16 were positive for WHV DNA at time point 0 and 6 weeks later (table 28) as were W360 and W414. WHV DNA and WHeAg levels are plotted individually for each animal allowing closer examination of the variation in parameter levels, particularly WHV DNA. The degree of variation around the mean WHV DNA level observed in all animals ranged from 0-50% between two consecutive points.

W406 and W407 were animals who had recovered from an infection and had no detectable WHV DNA or WHeAg and were anti-WHc negative. When W406 was treated the T cell response to HBcAg increased above 4 although, in response to PHA the S.I. decreased (figure 60). The opposite was true for W407 (figure 61) who sustained an increase in T cell responsiveness to PHA after treatment ceased, whereas the response to HBc continued to fluctuate. Both animals subsequently tested positive for anti-WHc and remained WHeAg negative.

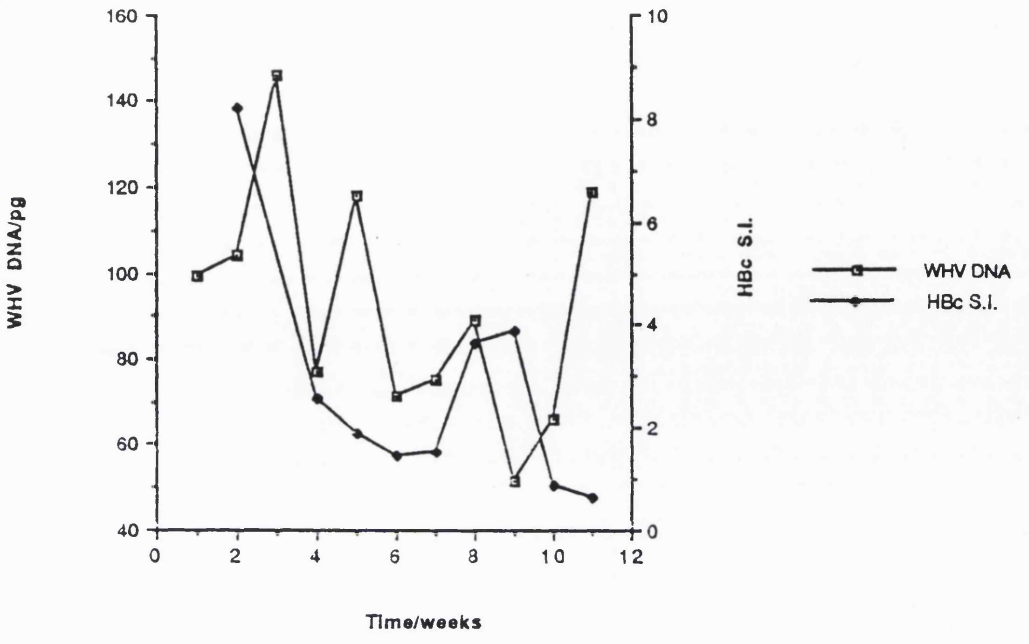


Figure 45. Changes in the WHV DNA levels and T cell S.I.s in response to HBcAg in W360, an untreated (control) chronic carrier animal.

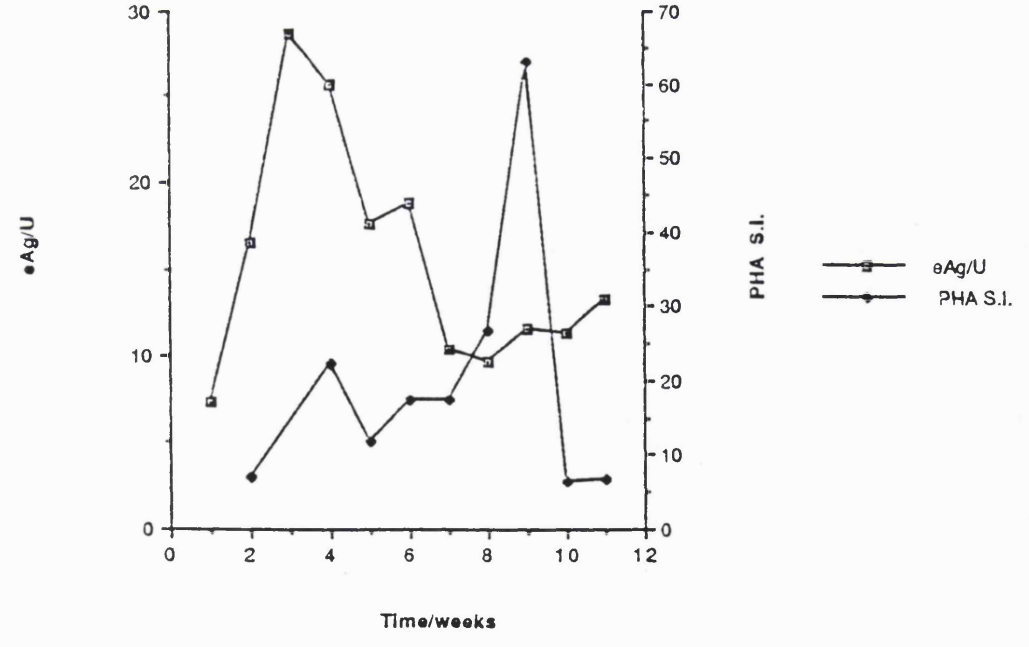


Figure 46. Changes in the WHeAg levels and T cell S.I.s in response to PHA in W360, an untreated (control) chronic carrier animal.

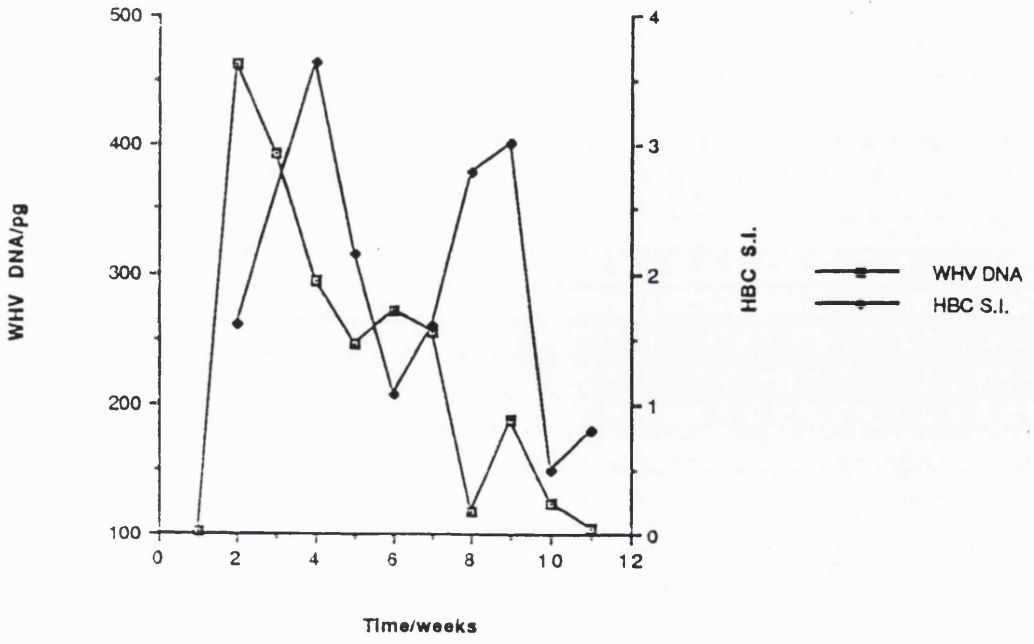


Figure 47. Changes in the WHV DNA levels and T cell S.I.s in response to HBcAg in W414, an untreated (control) chronic carrier animal.

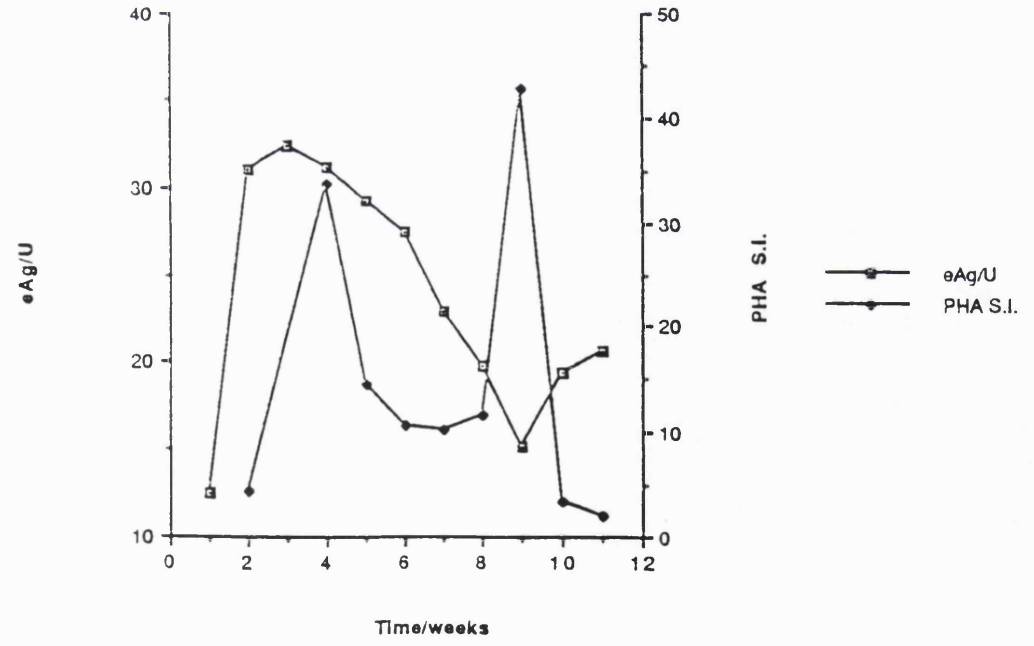


Figure 48. Changes in the WHeAg levels and T cell S.I.s in response to PHA in W414, an untreated (control) chronic carrier animal.

4.4.2. Animals Treated with 20mg/kg Tucaresol Only

A large degree of variation was seen in all 3 parameters (figures 49-52) for both treatments in all animals and at no time point was WHV DNA cleared from the serum. The GGT levels for W933 over the treatment period decreased before rising again once treatment ceased (figure 57).

4.4.3. Animals Treated with 20mg/kg Tucaresol and HBeAg

A large degree of variation was seen in all 3 parameters (figures 53-56) for both treatments in all animals and at no time point was WHV DNA cleared from the serum. The GGT level for both animals remained low during the first treatment (figure 58) (levels $\geq$ 10 indicate tumour). The GGT levels over the second treatment period for W936 revealed a steady decrease as seen in figure 58.

4.4.4. Animals Treated with 30mg/kg Tucaresol Only

W520 and W527 developed large abscesses at the site of injection and therefore treatment was stopped.

4.4.5. Animals Treated with 40mg/kg Tucaresol Only

At no time point was WHV DNA cleared from the serum and a large degree of variation was observed in all parameters (figures 62-65). In both animals treatment was stopped after 1 week due to abscess formation.

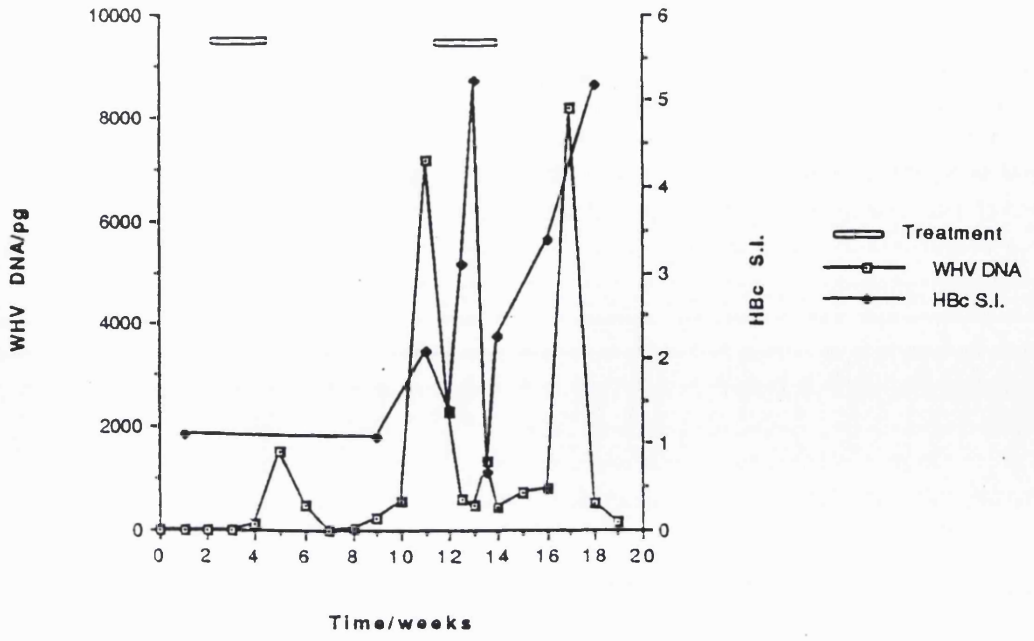


Figure 49. Effect of 20mg/kg tucaresol on the WHV DNA level and T cell response to HBcAg in W933

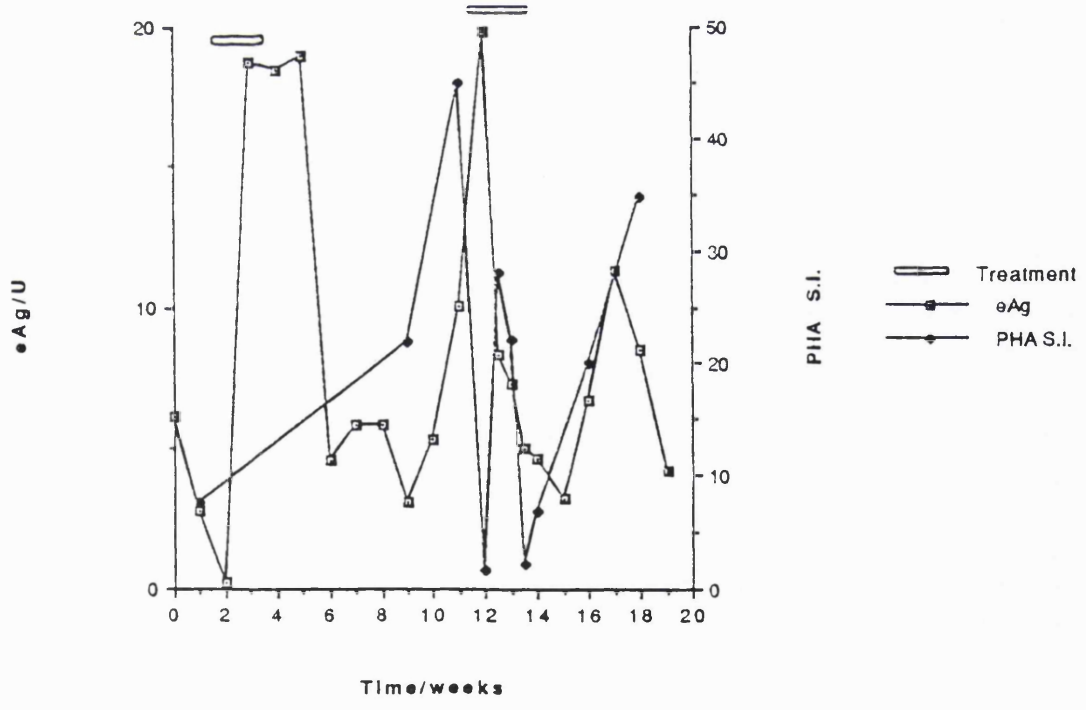


Figure 50. Effect of 20mg/kg tucaresol on the WHeAg level and T cell response to PHA in W933

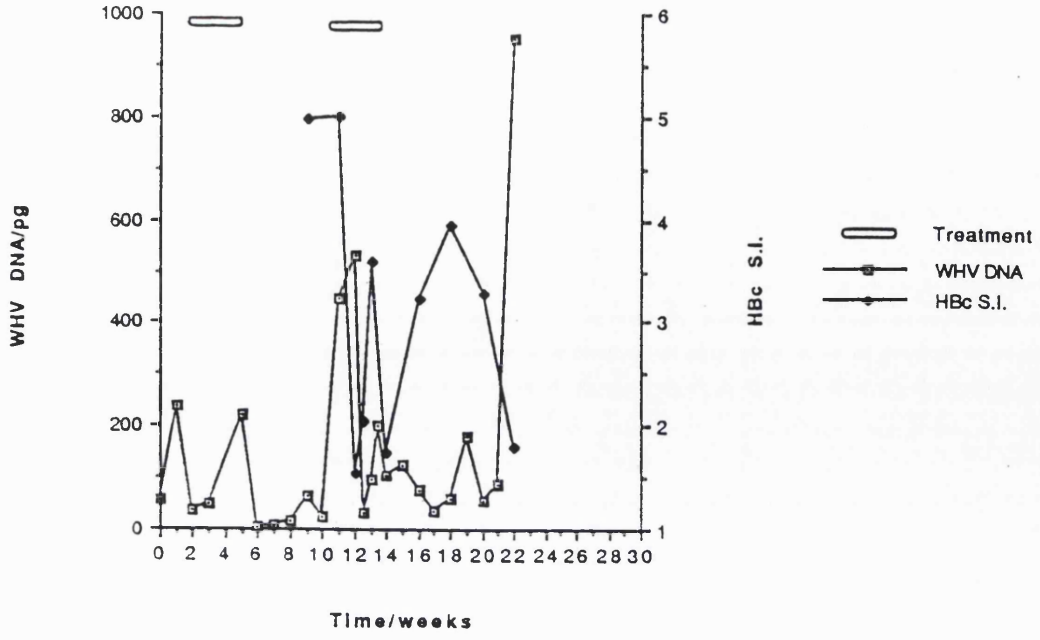


Figure 51. Effect of 20mg/kg tucaresol on the WHV DNA level and T cell response to HBcAg in W937

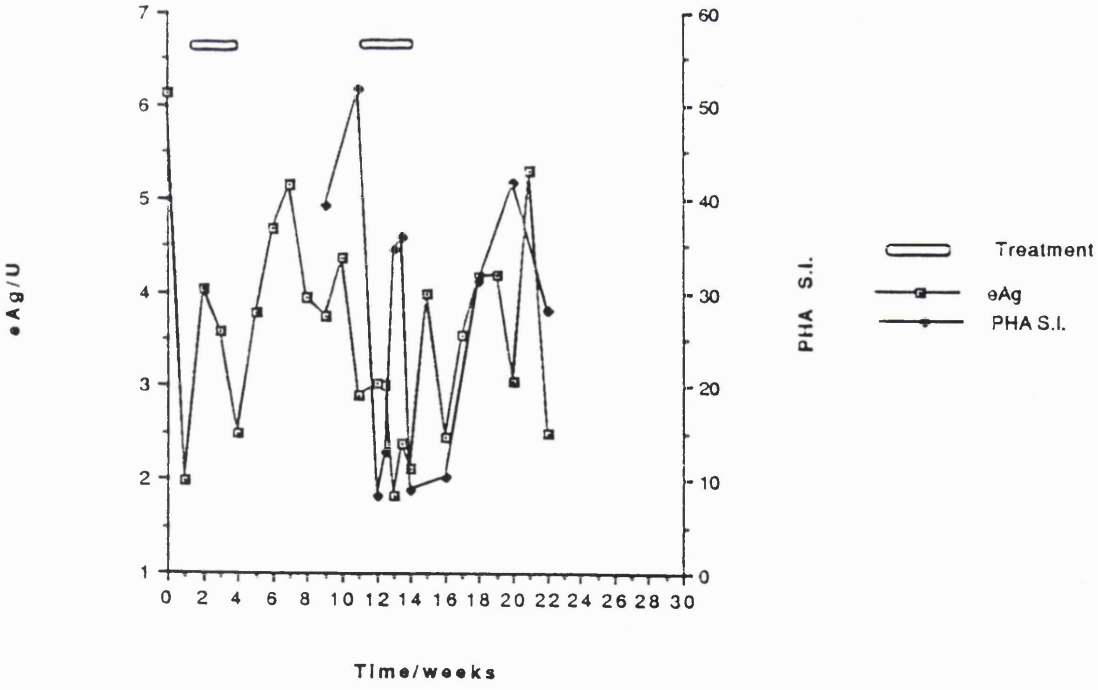


Figure 52. Effect of 20mg/kg tucaresol on the WHeAg level and T cell response to PHA in W937

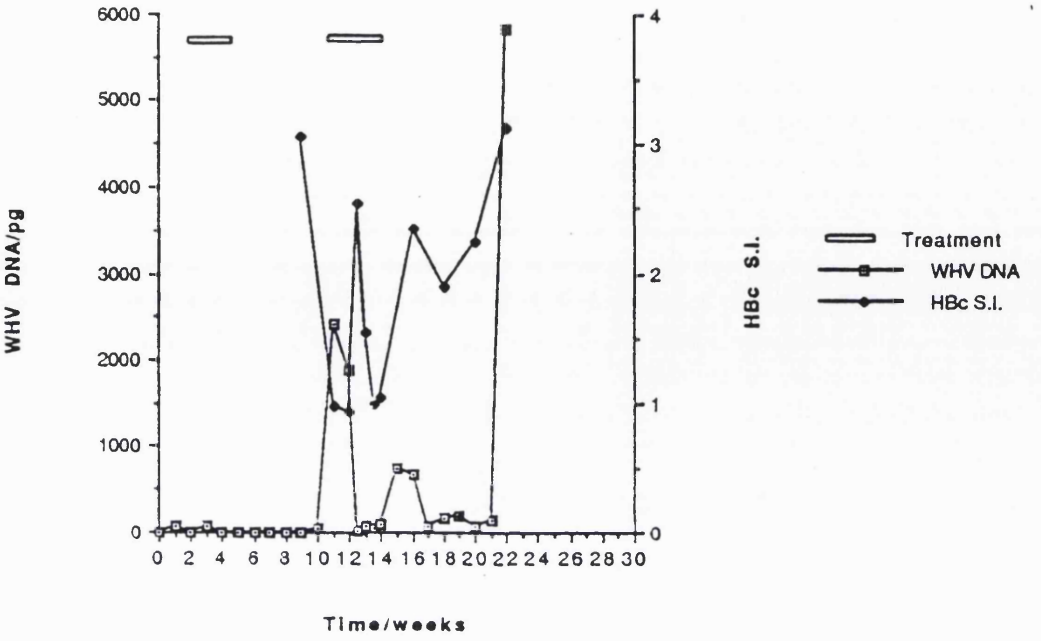


Figure 53. Effect of 20mg/kg tucaresol and HBeAg on the WHV DNA level and T cell response to HBcAg in W935

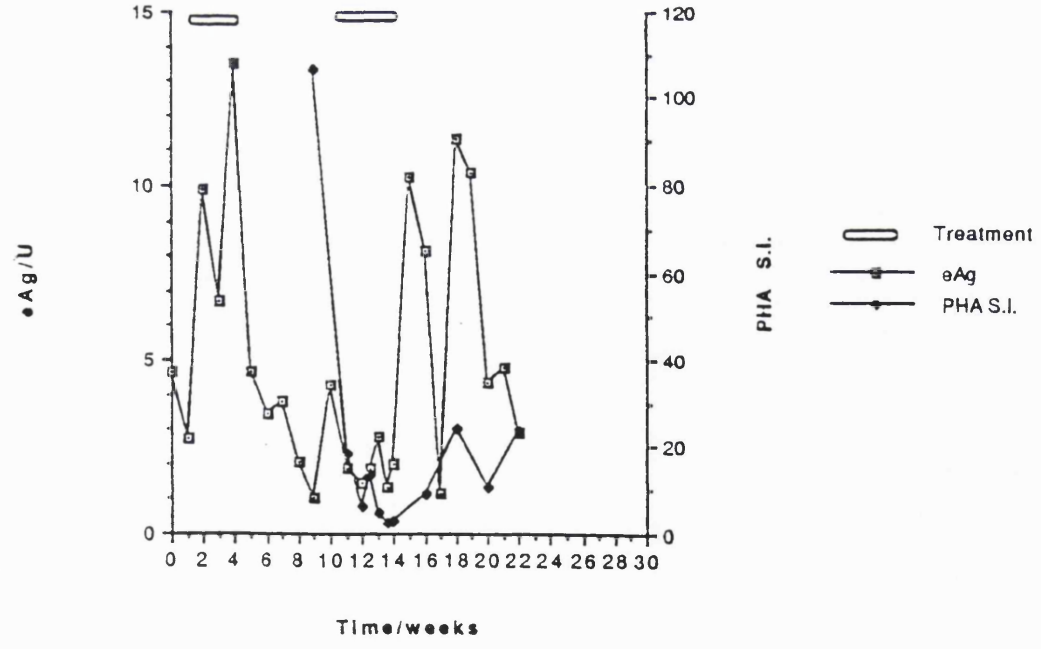


Figure 54. Effect of 20mg/kg tucaresol and HBeAg on the WHeAg level and T cell response to PHA in W935

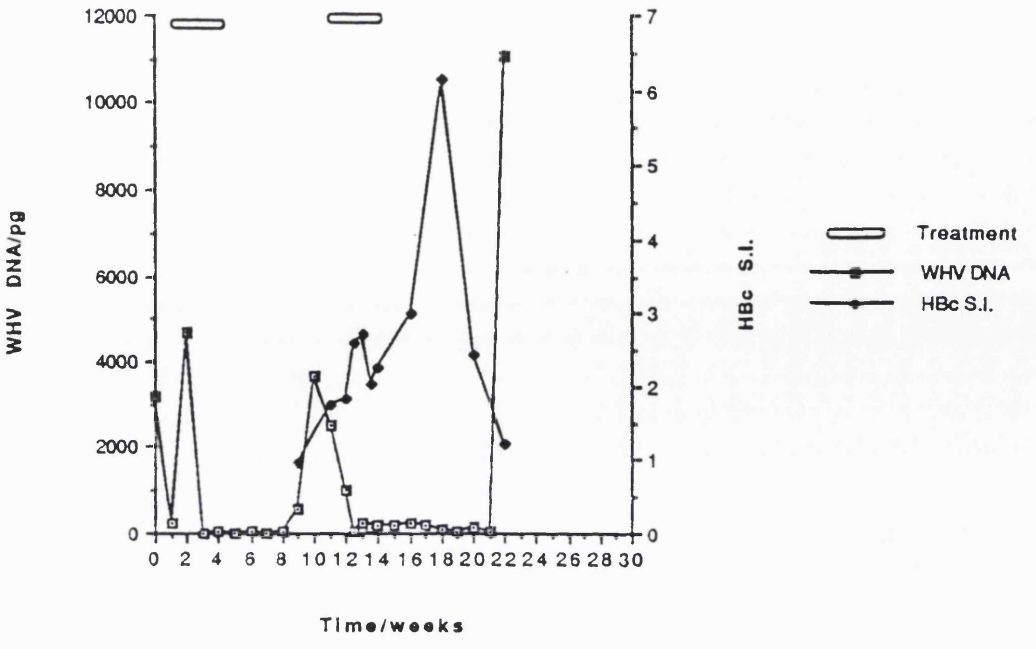


Figure 55. Effect of 20mg/kg tucaresol and HBeAg on the WHV DNA level and T cell response to HBcAg in W936

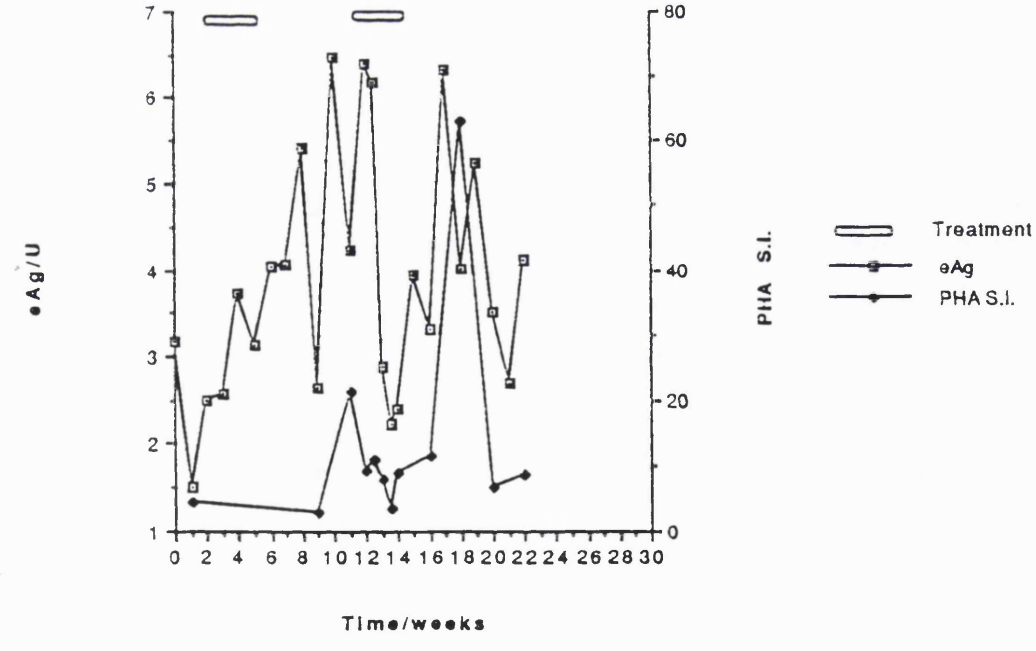


Figure 56. Effect of 20mg/kg tucaresol and HBeAg on the WHeAg level and T cell response to PHA in W936

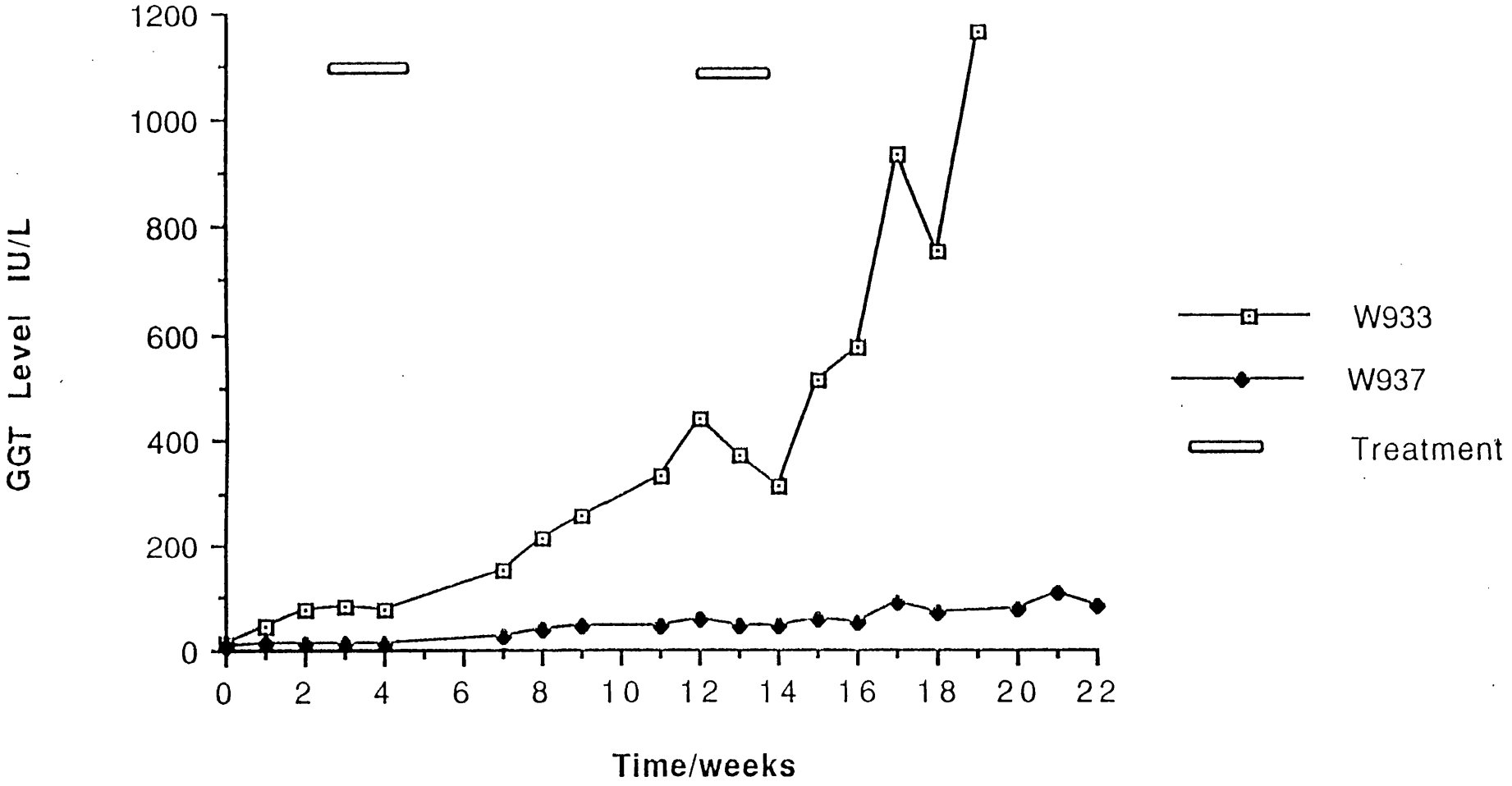


Figure 57. GGT Levels for Animals treated with 20mg/kg tucaresol only.

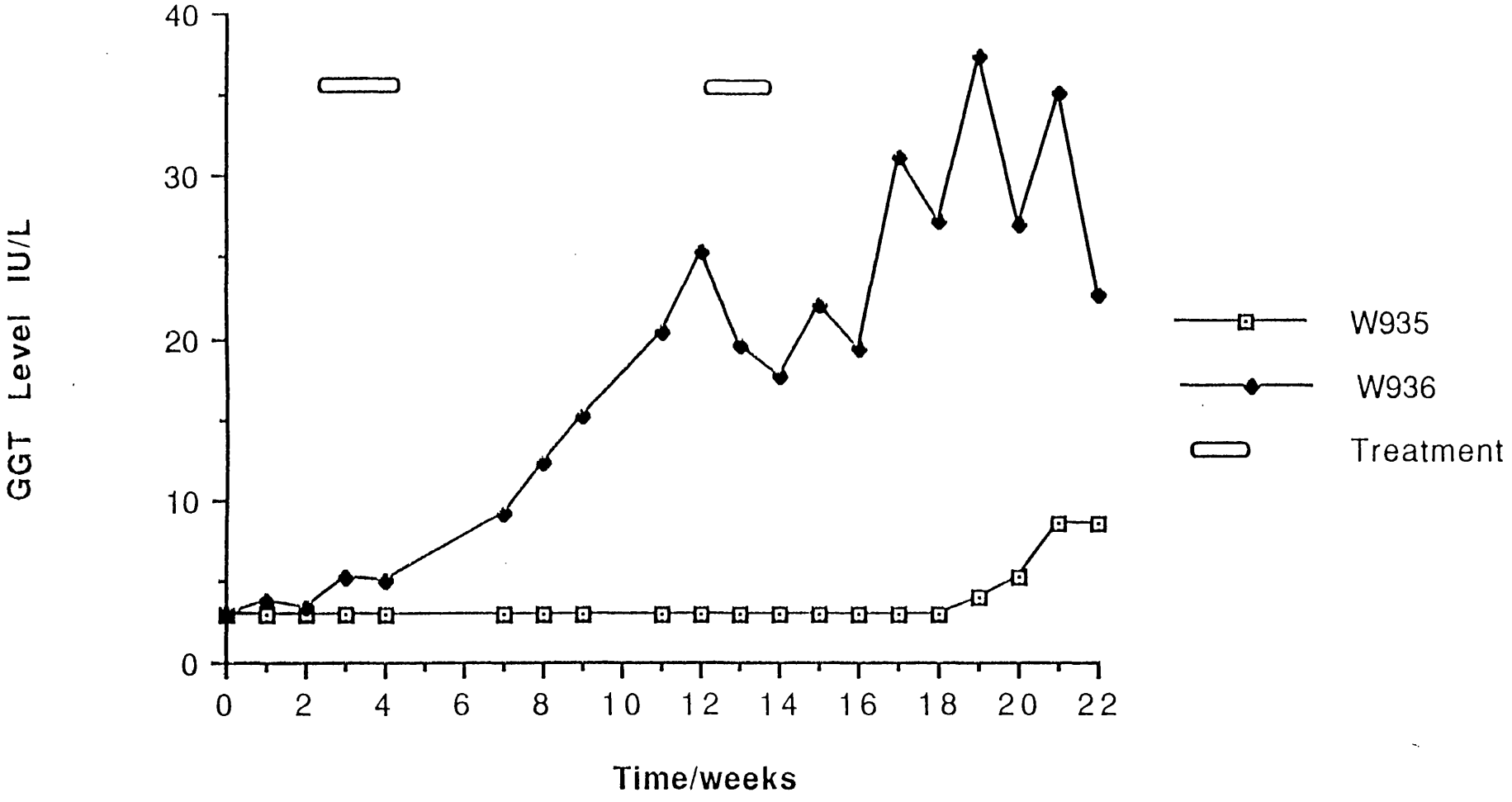


Figure 58. GGT Levels for Animals treated with 20mg/kg tucaresol and HBeAg.

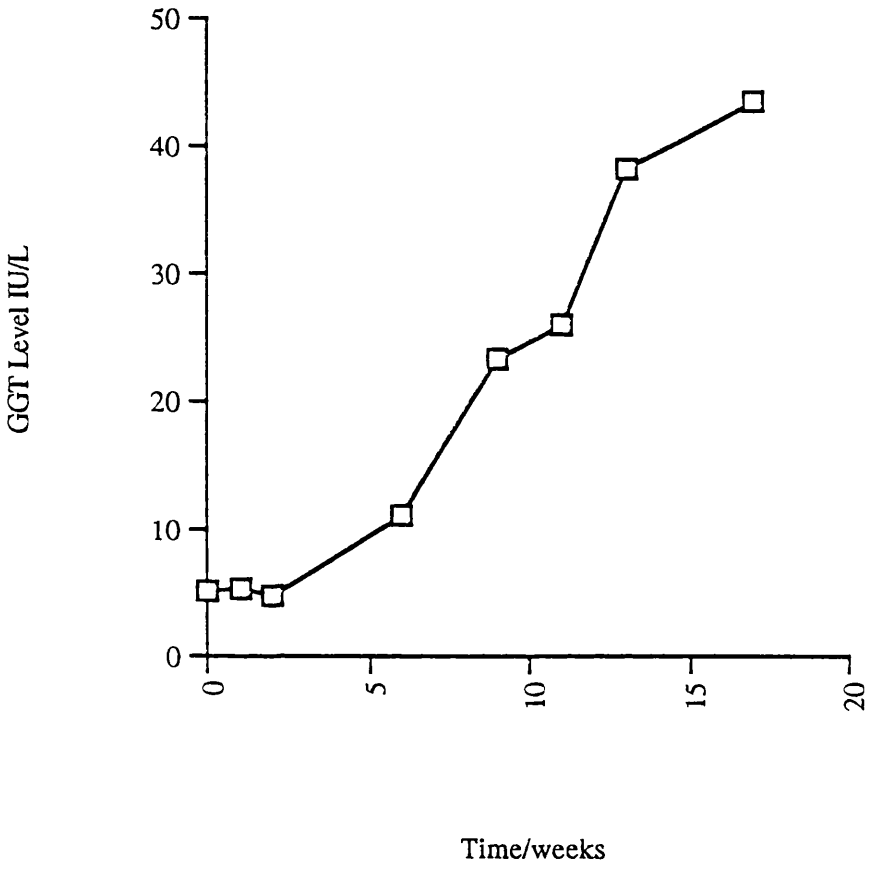


Figure 59. GGT Levels for W754, an untreated animal

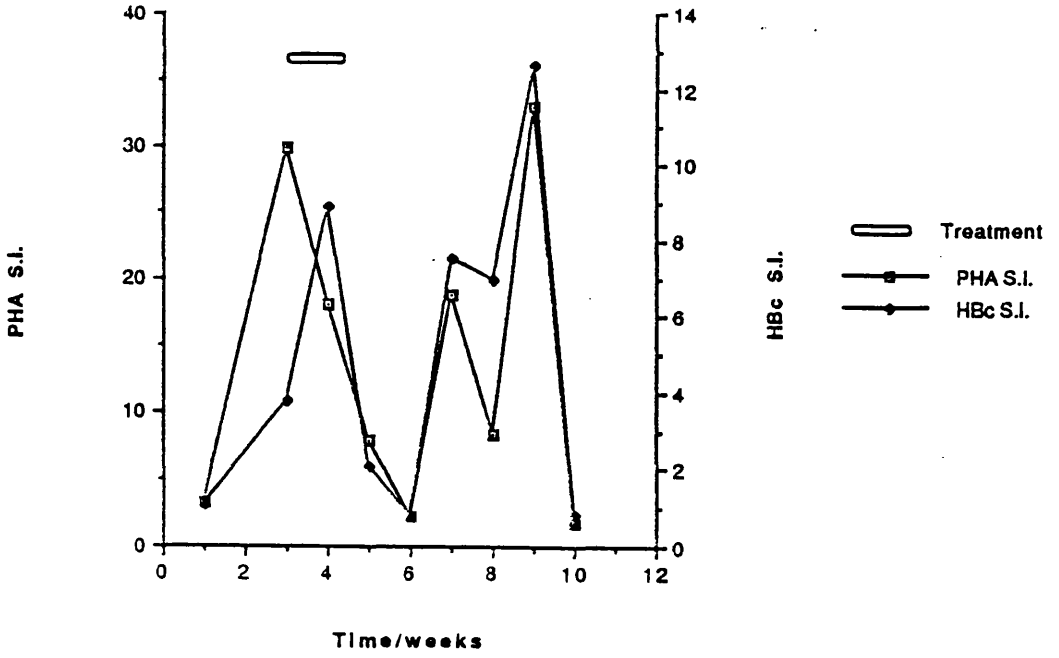


Figure 60. Effect of 30mg/kg tucaresol and HBeAg on the T cell response to HBcAg and PHA in W406, an animal who had recovered from infection

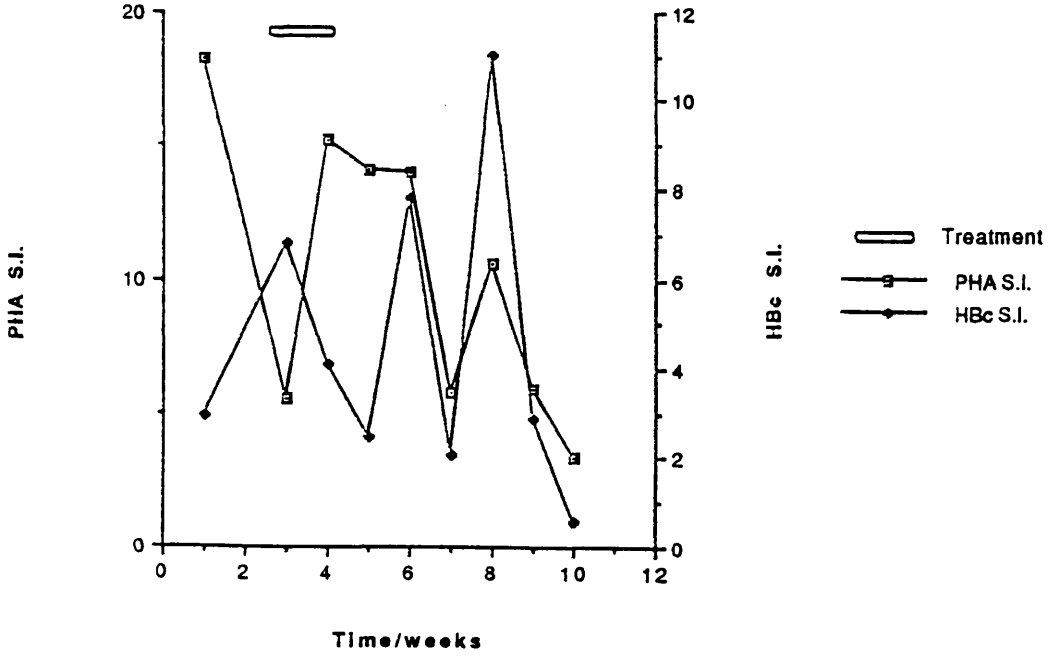


Figure 61. Effect of 30mg/kg tucaresol and HBeAg on the T cell response to HBcAg and PHA in W407, an animal who had recovered from infection

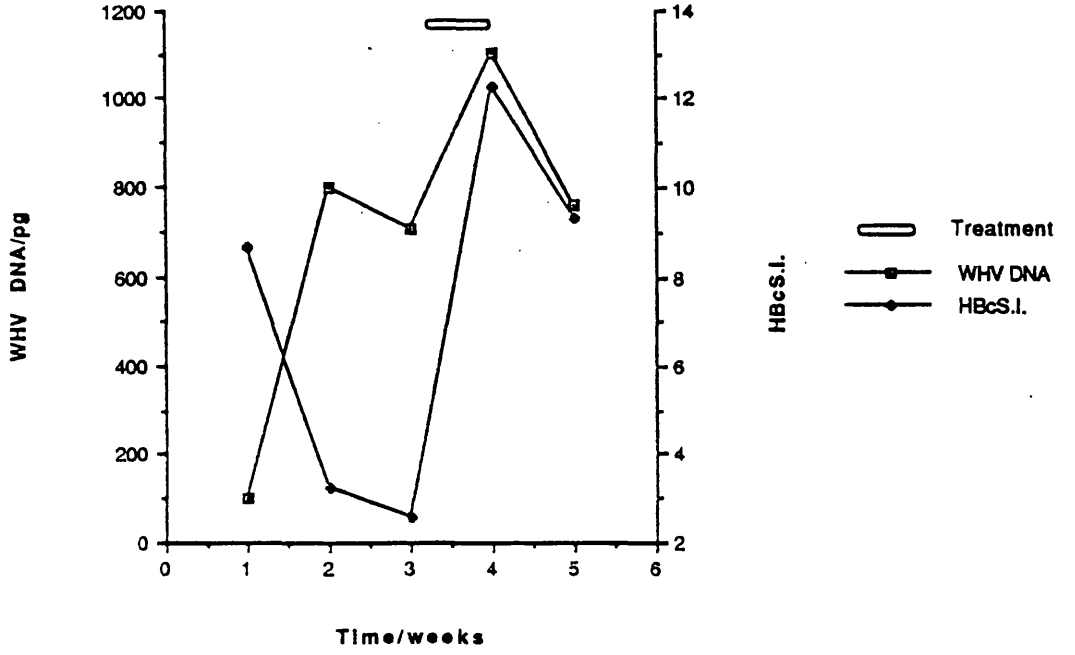


Figure 62. Effect of 40mg/kg tucaresol on the WHV DNA level and T cell response to HBcAg in W507

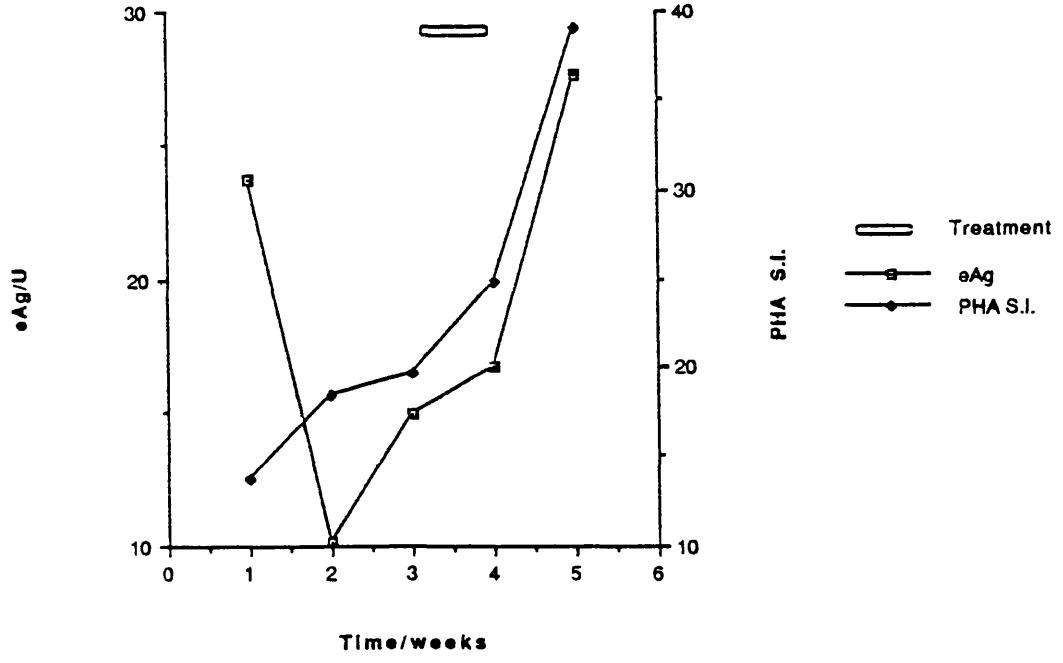


Figure 63. Effect of 40mg/kg tucaresol on the WHeAg level and T cell response to PHA in W507

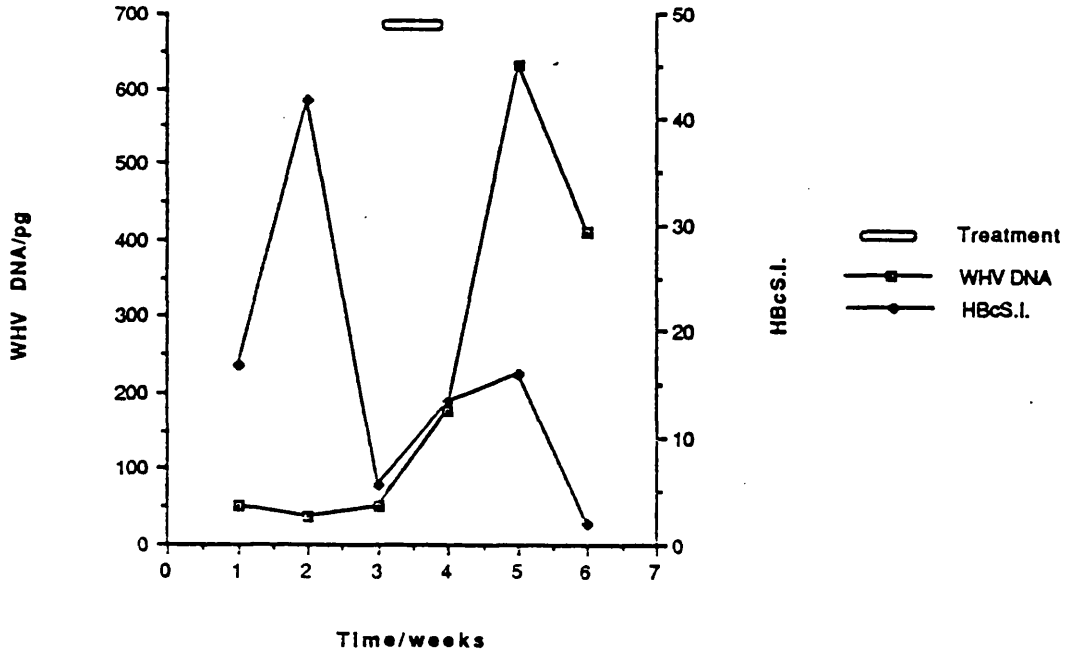


Figure 64. Effect of 40mg/kg tucaresol on the WHV DNA level and T cell response to HBcAg in W518

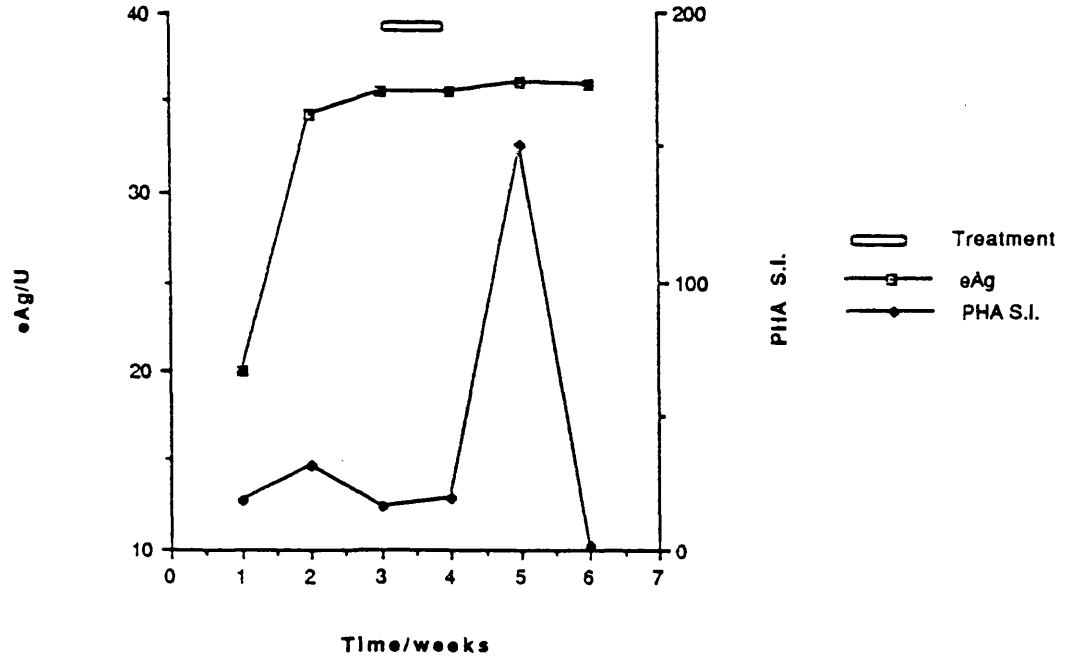


Figure 65. Effect of 40mg/kg tucaresol on the WHeAg level and T cell response to PHA in W518

4.4.6. Animals Treated with 40mg/kg Tucaresol and HBeAg

A large degree of variation was seen in all parameters and at no time point was WHV DNA cleared from the serum (figures 66-69). Treatment was stopped after 1 week due to abscess formation.

W405 showed a decrease in WHV DNA levels whilst on treatment and after and continues to eradicate DNA from the serum (figure 70). The WHeAg levels confirm the drop in viral replication (figure 71). The T cell response to both PHA and HBcAg increases. The time of increase in T cell response coincides with the drop in WHV DNA and eAg levels. W405 was sacrificed at week 8 due to abscess formation.

W363 shows no evidence of an effect of the drug and there was a large degree of variation in all parameters (figures 72 and 73). At no time point was WHV DNA cleared from the serum.

4.4.7. Statistical Analysis

Only 1 animal (W405) cleared WHV DNA from the serum after 6 weeks (table 28). WHV DNA was detected in the serum of all the other treated animals after 6 weeks. There is no significant difference between WHV DNA levels of animals treated with tucaresol only and tucaresol plus HBeAg ($p=0.08$) (table 29). There is also no significant difference between untreated animals and tucaresol only treated animals

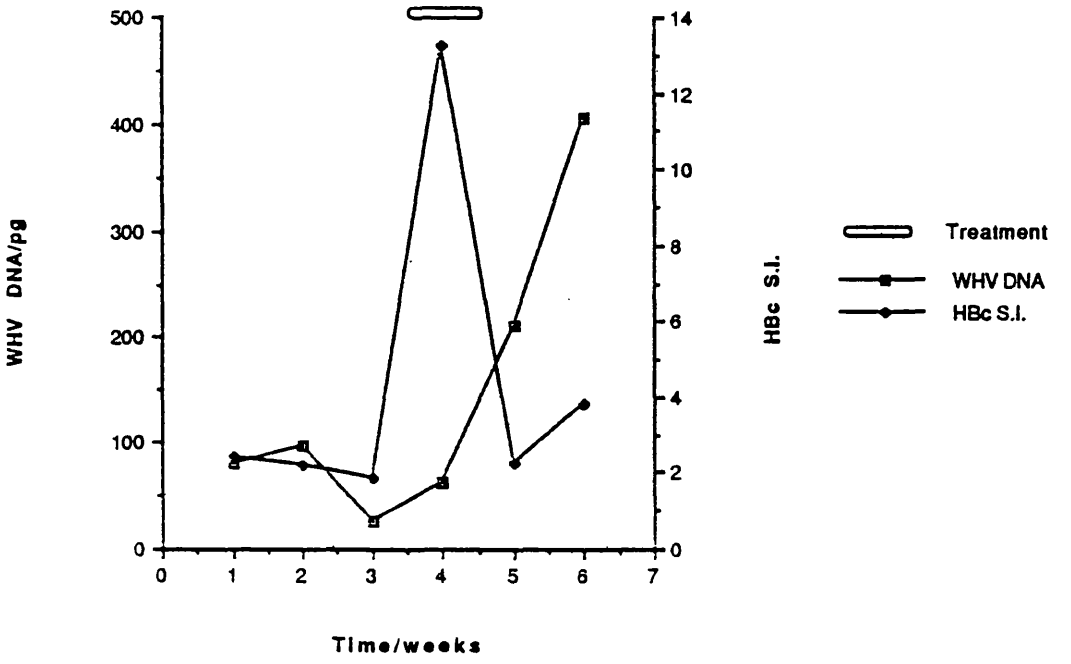


Figure 66. Effect of 40mg/kg tucaresol and HBeAg on the WHV DNA level and T cell response to HBcAg in W360

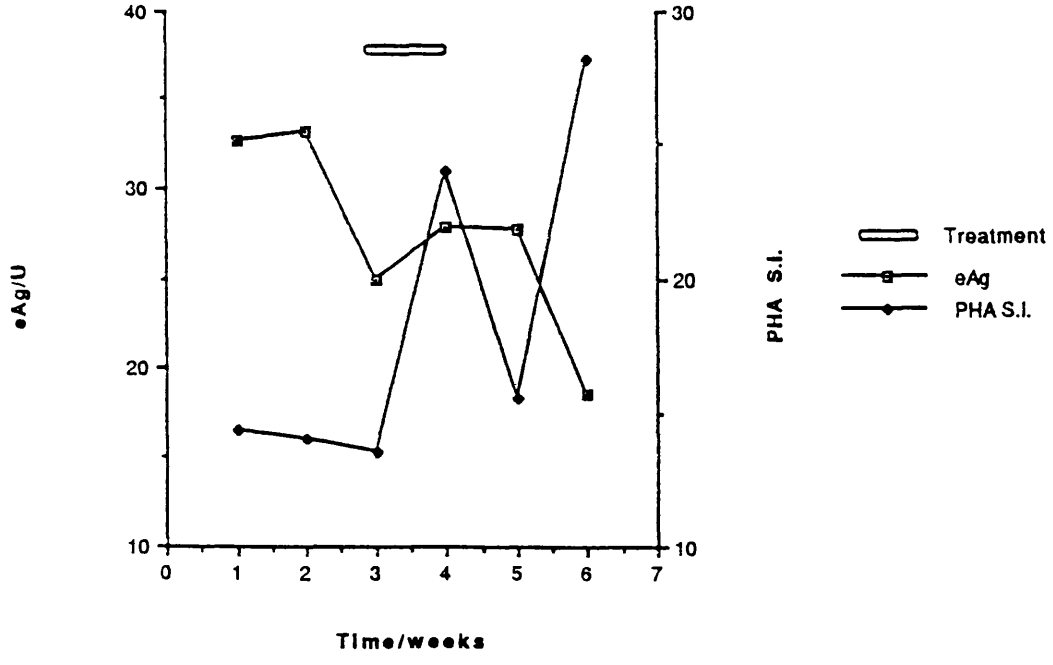


Figure 67. Effect of 40mg/kg tucaresol and HBeAg on the WHeAg level and T cell response to PHA in W360

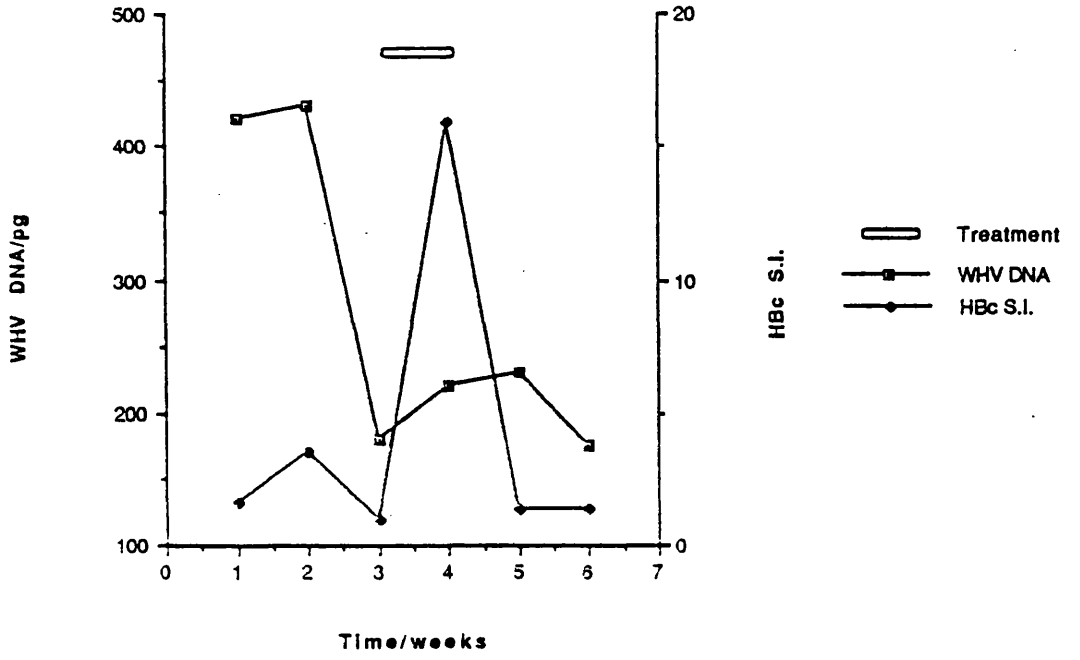


Figure 68. Effect of 40mg/kg tucaresol and HBeAg on the WHV DNA level and T cell response to HBcAg in W414

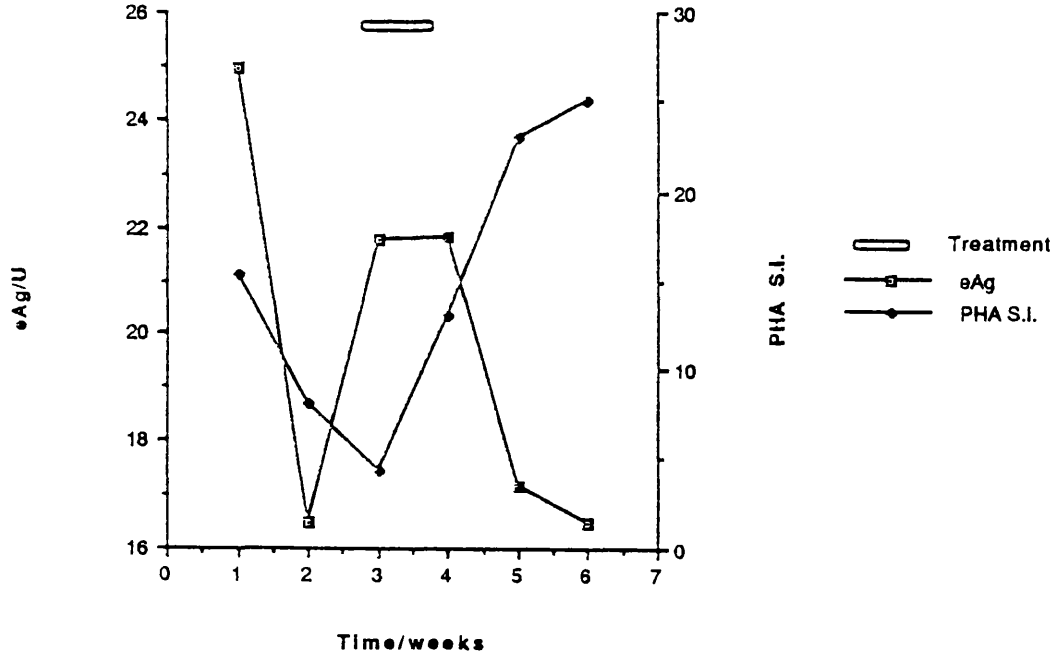


Figure 69. Effect of 40mg/kg tucaresol and HBeAg on the WHeAg level and T cell response to PHA in W414

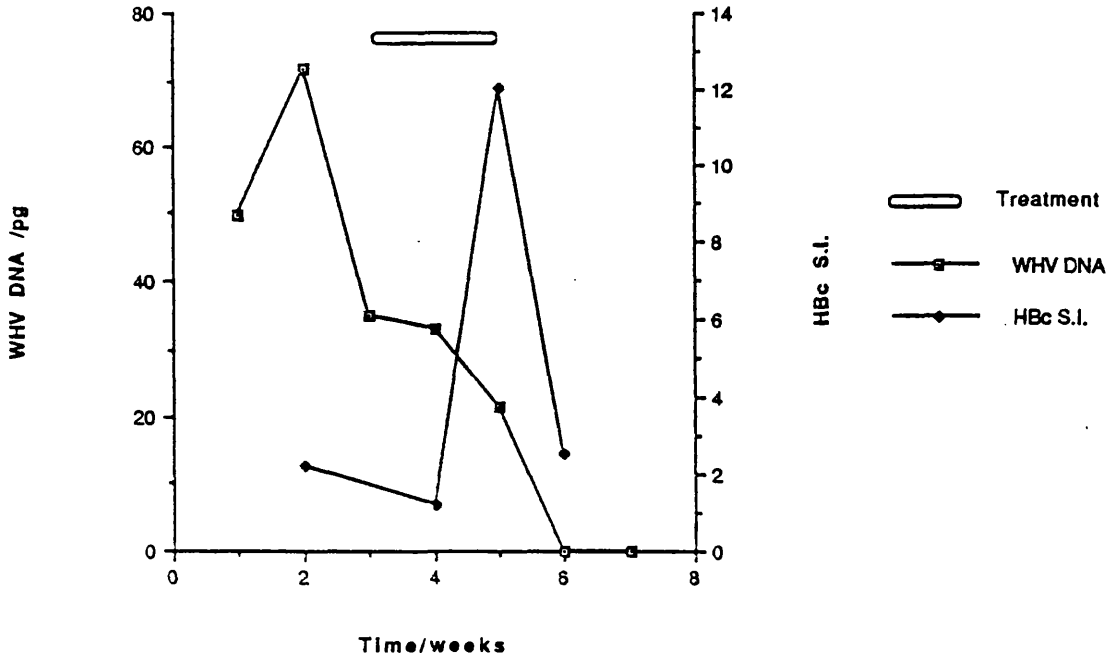


Figure 70. Effect of 40mg/kg tucaresol and HBeAg on the WHV DNA level and T cell response to HBcAg in W405

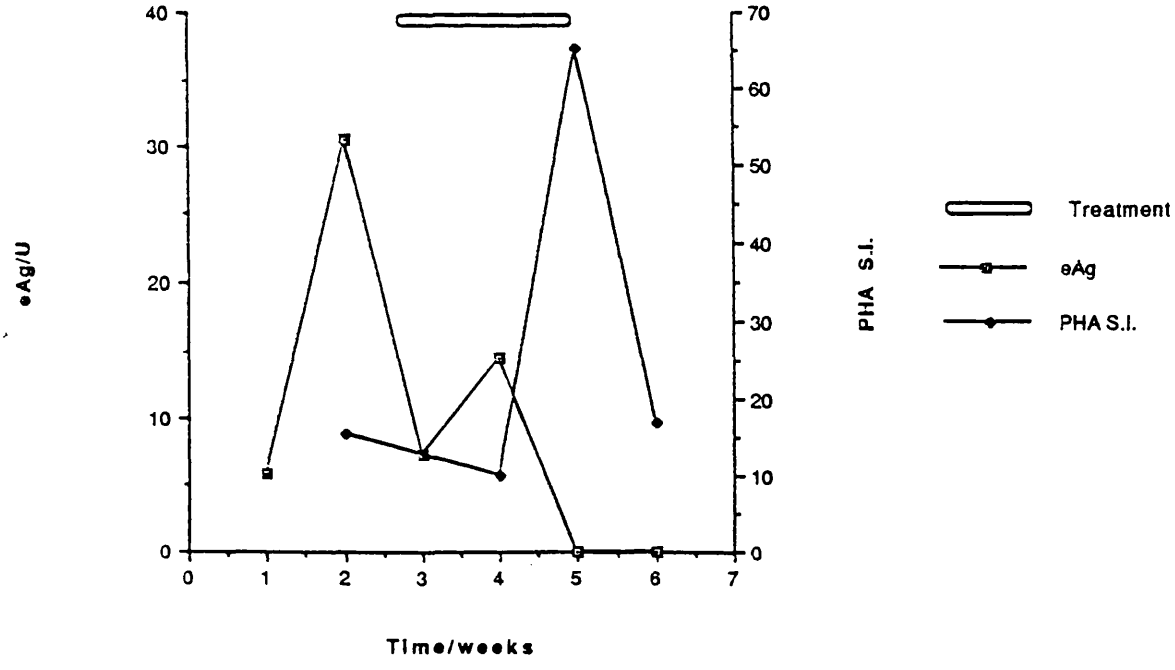


Figure 71. Effect of 40mg/kg tucaresol and HBeAg on the WHeAg level and T cell response to PHA in W405

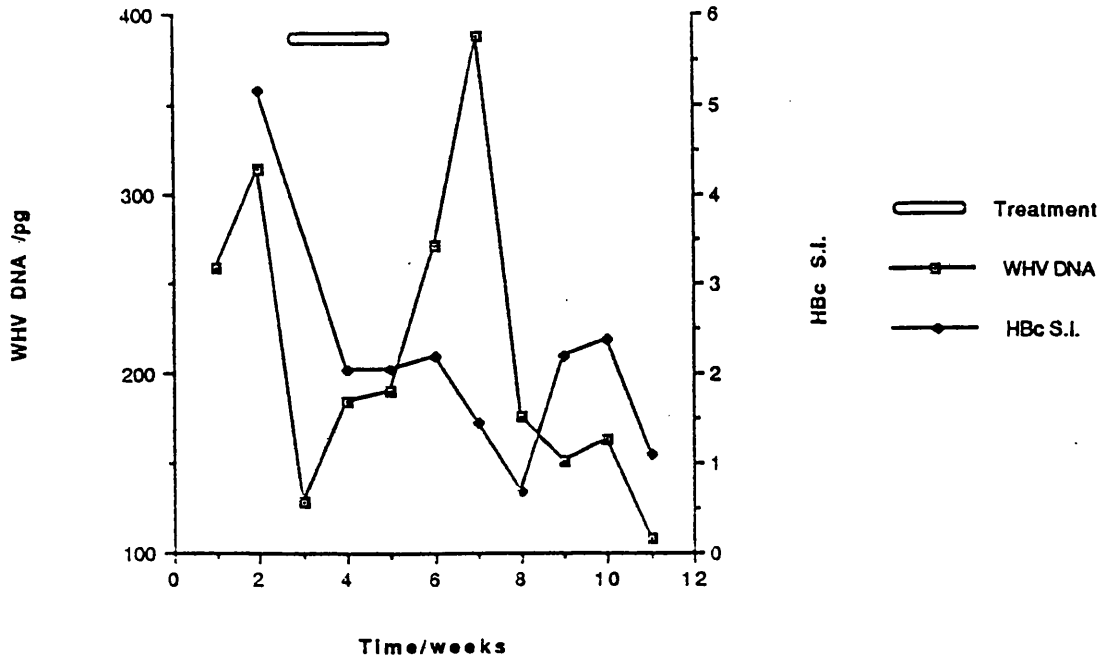


Figure 72. Effect of 40mg/kg tucaresol and HBeAg on the WHV DNA level and T cell response to HBcAg in W363

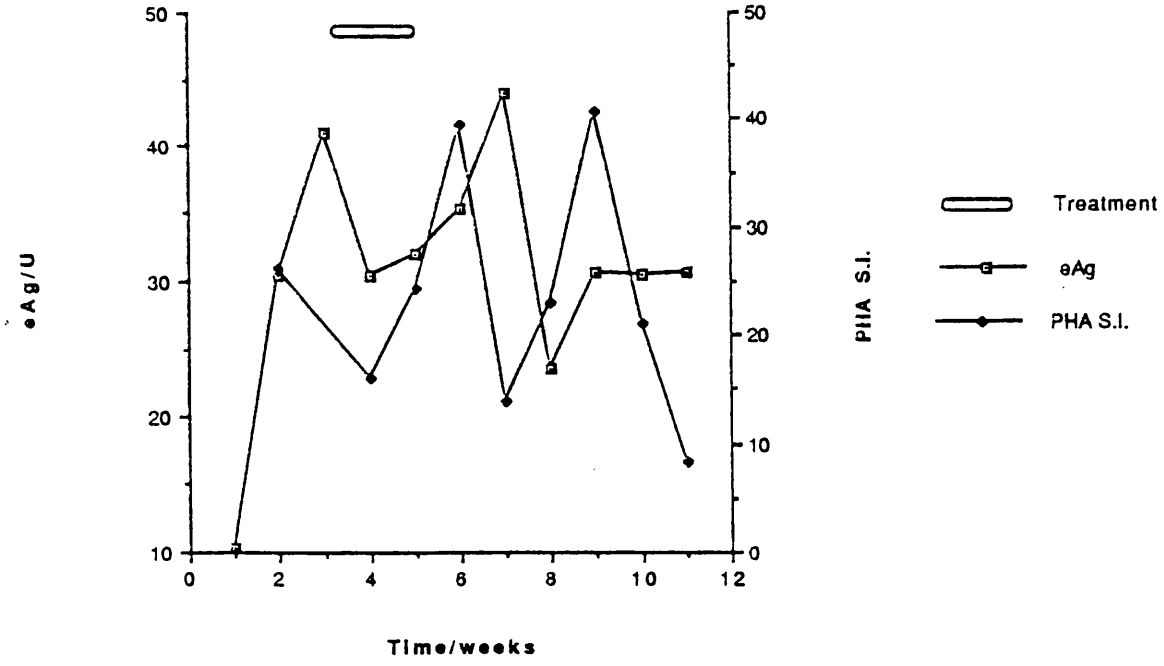


Figure 73. Effect of 40mg/kg tucaresol and HBeAg on the WHeAg level and T cell response to PHA in W363

(p=0.81) or between untreated animals and tucaresol plus HBeAg treated animals (p=0.11). p values <0.05 were considered significant.

4.4.8 Side Effects

Woodchucks given the lower dose of 20mg/kg were found to develop abscesses which were attributed to the large volume of the drug. However, once the volume was reduced to 2mls and 1ml abscesses still occurred at the higher doses.

Both W363 and W405 developed an abscess. The abscess on W363 appeared on the leg of injection and appeared to start healing once treated with topical oxytetracycline and betadine. However, the wound reopened and was subsequently slow to heal. Another abscess developed in the front leg after the site of injection was changed. The woodchuck eventually recovered after 6 weeks but the abscess on the leg did not heal completely until a few weeks later.

W405 also developed an abscess after 1 week on treatment which was treated with antibiotic. It deepened after 2 weeks on treatment. After cessation of drug treatment no deterioration was observed the following week. However, during week 8 the leg of injection (right hind leg) started to bleed, became severely indurated and eventually immobile. The animal was unable to move and appeared to have difficulty breathing. White cells were visible in the anterior chamber of the left eye, possibly due to the effects of the inflammation. The animal was therefore culled at week 8. The spleen was found to be enlarged (9cms x 2cms) and all cells cultured from it became infected

with bacteria (attributed to the cells and not to the culture components) which was thought to be derived from the cell preparation.

Study 2. MPC-866

W935 began with a GGT level above 45 which indicates the development of HCC. This level dropped to less than 40 and was sustained whilst on treatment but rebounded once treatment was stopped (figure 74). W936 showed the same pattern but started with a lower GGT level of 19 which dropped to 15 and was maintained during treatment (figure 75). Figure 59 shows the progression of GGT levels in an untreated animal with HCC over a period of 4 months.

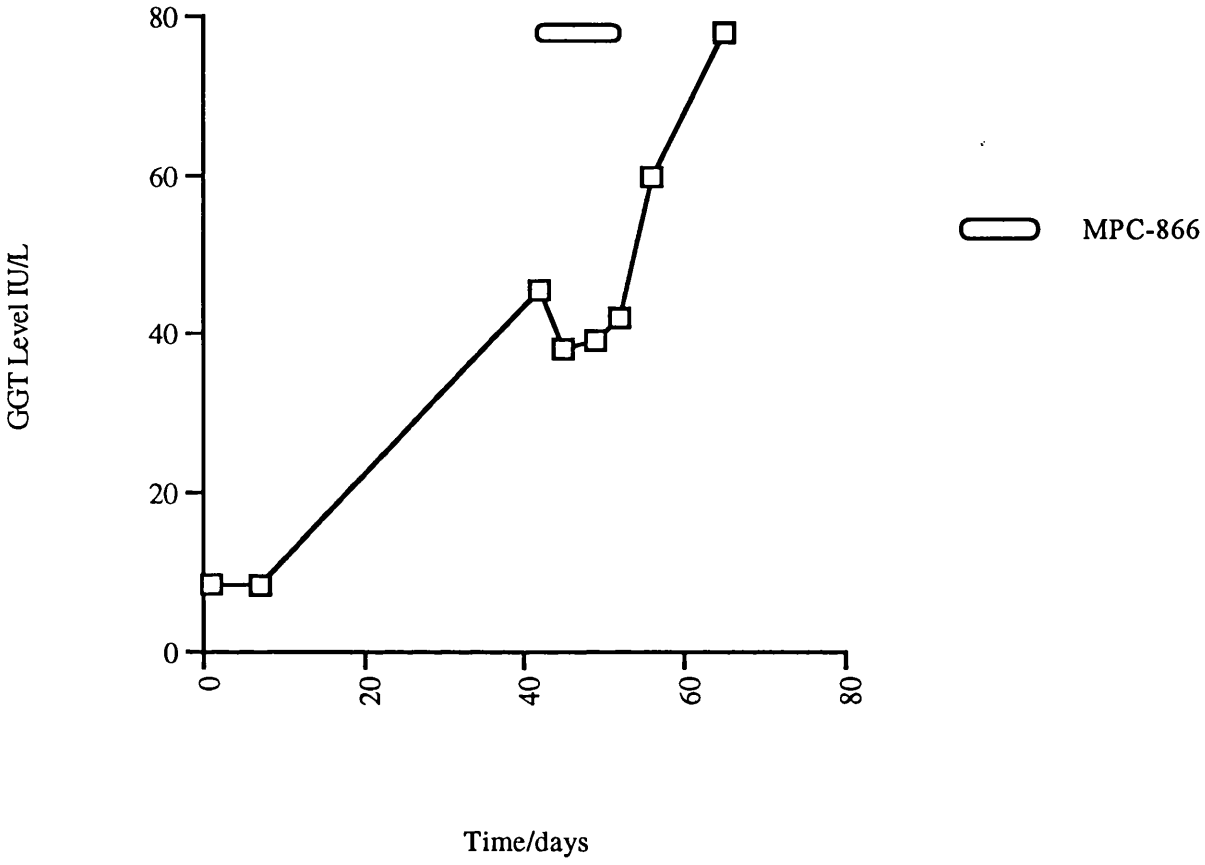


Figure 74. Variation in Gamma GT Levels in W935, a chronic carrier with a tumour treated with MPC-866

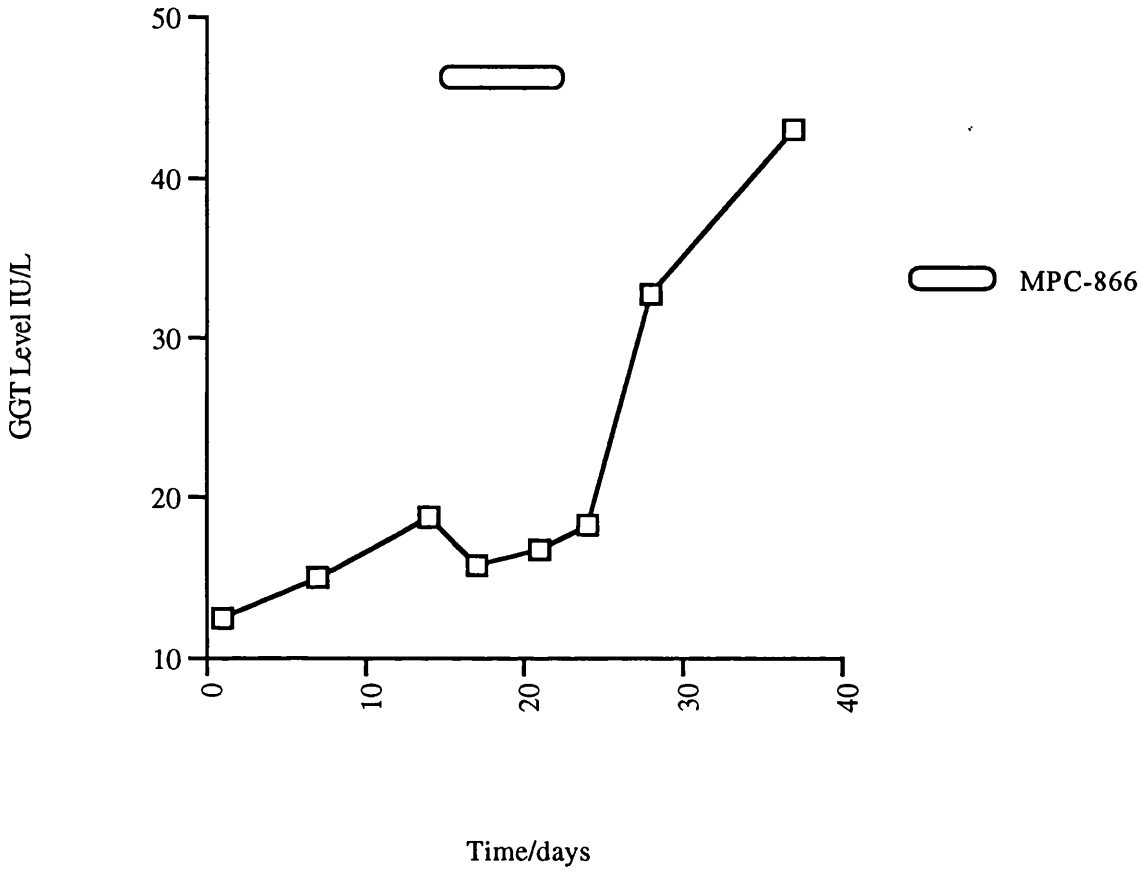


Figure 75. Variation in Gamma GT Levels in W936, a chronic carrier with a tumour treated with MPC-866

Animal Number	Group 1/2/3	Presence (+) or absence (-) of WHV DNA at week 0	Presence (+) or absence (-) of WHV DNA at week 6
W1	1	+	+
W5	1	+	+
W10	1	+	+
W16	1	+	+
W360	1	+	+
W414	1	+	+
W933 (treatment 1)	2	+	+
W937 (treatment 1)	2	+	+
W933 (treatment 2)	2	+	+
W937 (treatment 2)	2	+	+
W507	2	+	+
W518	2	+	+
W935 (treatment 1)	3	+	+
W936 (treatment 1)	3	+	+
W935 (treatment 2)	3	+	+
W936 (treatment 2)	3	+	+
W360	3	+	+
W414	3	+	+
W363	3	+	+
W405	3	+	-

Table 28. Summary of the WHV DNA levels in untreated (group 1), tucaresol only treated (group 2) and tucaresol plus HBeAg treated (group 3) woodchucks at week 0 and 6 weeks later.

Statistics	WHV DNA levels in the Untreated Group/pg (n=6)	WHV DNA levels in the Tucaresol only treated group/pg (n=6)	WHV DNA levels in the Tucaresol plus HBeAg treated group/pg (n=8)
	70	500	30
	260	1200	700
	250	210	46
	860	100	100
	1398	750	400
	960	400	170
			0
			265
Mean	633	526.67	213.88
S.E.M.	518.94	400.58	237.91

Table 29. WHV DNA levels of all animals after 6 weeks.

	Tucaresol only treated	Tucaresol plus HBeAg treated
Untreated	p= 0.81	p= 0.11
Tucaresol plus HBeAg treated	p= 0.08	

Table 30. Mann-Whitney U-test p values comparing each of the three groups using data from table 29. p values <0.05 were considered significant.

4.5 Discussion

Previous work carried out by the Wellcome Foundation found that there was no enhancement of the PBL response to PHA or WHV core antigen in animals treated at the lower dose of 10mg/kg Tucaresol. They did observe a drop in viral load in one of two monkeys infected with SIV treated at the higher dose of 30mg/kg. A dose range study was undertaken beginning with 20mg/kg and rising to 40mg/kg with the aim of causing the eradication of WHV DNA from the serum.

The WHV DNA dot blot was assessed for the level of interassay variation in order to ensure that changes associated with therapy were real. The results obtained from two separate blots using the same samples proved that whilst absolute values varied by 20% the pattern of fluctuation remained the same. This eliminated one area of doubt regarding the validity of the results. WHeAg crossreacts with HBeAg and the commercial assay was pre-tested using serum from infected and uninfected animals which found that the presence of WHeAg coincided with the presence of WHV DNA. In addition the T cell responses were assessed using previous conditions and the s.e.m. of the triplicate values did not exceed 20%.

The results are difficult to interpret mainly because of the large amount of variation seen in all four parameters. The same variation in T cell response is seen in untreated animals and therefore a direct T cell effect is difficult to conclude. The blood samples were taken at weekly time points during the first treatment which may have

overlooked an early change in WHV DNA levels; as a result blood was sampled biweekly during the second treatment. In animals treated with 20mg/kg the variation in WHV DNA levels ranged from 900-11000 pg which was very high in comparison to the range of variation observed in untreated animals (100-400 pg) and in those animals treated with 40mg/kg (50-1000pg). There is no sustained loss of WHV DNA from the serum in any of the animals. At the higher dose of 40mg/kg WHV DNA levels drop markedly in W405 until death. The added complication in this animal is the development of an abscess which progressed and ultimately killed the animal due to secondary infection.

The two recovered woodchucks given 30mg/kg tucaresol and HBeAg showed an increased T cell response to HBcAg. This may indicate a more specific augmentation of T cell activity. The development of anti-WHc antibodies indicates that this strategy induces the immune response.

The consistent drop in GGT levels over the treatment period in W933 and W936, treated with 20mg/kg, suggests that the drug had an anti-tumour effect which had previously been reported for mouse adenocarcinoma (Jenkins 1993). If this was the case, events leading to regression of tumour growth may have been dependent on Th cells as already suggested.

I.M. injection of the drug may not be the best route of administration but no other alternative exists for these animals. The animals were clearly distressed on occasions

when the drug was administered and whether this was due to injection near a nerve or the drug causing a stinging effect is unclear. The drug was made up in a smaller volume because a larger volume produced large abcesses as reported before. Due to the small volume, the pH could not be adjusted with HCl as precipitation resulted. It is clear that this drug causes the formation of abscesses which , because of the nature and behaviour of the animals, can be susceptible to secondary infections. However, in humans the oral route is proposed which may circumvent these problems.

In conclusion, at the maximum tolerated dose of tucaresol no clearance of WHV DNA was observed. Furthermore there was no significant difference between those animals given HBeAg with the drug and those given tucaresol alone. It does not therefore seem reasonable to continue treatment in man. As a result of the large variation observed in all parameters the woodchuck may not be an appropriate model in which to study the action of drugs which do not result in a complete ablation of markers.

4.5.2. MPC-866

Administration of this drug I.M. did not produce any abscesses. This could be due to the small volume or the neutral pH. A drop in GGT levels was observed which may indicate an anti-tumour effect. In contrast there was a steady rise in GGT levels in an untreated animal with a developing tumour. No apparent side-effects were detected. We can speculate on the mechanism of action of this drug on WHV; it may bind to the primer-template groove of the reverse transcriptase and inhibit its activity which

causes a reduction in virion production or it may bind to covalent, closed, circular DNA molecules and prevent their transcription into mRNA and thereby infectious protein. Both of these mechanisms would result in a regression of tumour growth and hence a drop in the GGT levels. The variation seen in this study may have been affected by the outbred nature of these animals and any parasitic infections harboured by the animals under study may have had an effect on the immune response observed.

Chapter 5

The Adoptive Transfer of T Lymphocytes into WHV Persistently Infected Woodchucks

5.1. Introduction

The role of T cells in hepatitis B virus infection is thought to be pivotal in determining the outcome of infection. The infusion of T lymphocytes into patients as a form of therapy for viral diseases is currently under discussion and in some cases already in practice (Riddell et al 1992, Papadopoulos et al 1994). Several different approaches can be used - T cells can be removed and expanded non-specifically in vitro using IL-2 and then reinfused or they can be expanded specifically by antigens, with or without cytokines. These methods rely on administration of a single dose of cells. Another option is to reinfuse over a period of time using several doses which requires a constant source of T cells. No experiments of this kind have been attempted in a woodchuck and consequently several factors have not been addressed. Overcoming graft versus host disease requires information on the woodchuck MHC system which is not available. We circumvented this problem by infusing autologous cells. The number of cells to be reinfused must be determined and if regular T cell infusion is to be considered the establishment of a T cell line is essential.

In addition, IL-12 (cytotoxic lymphocyte maturation factor, natural killer cell stimulatory factor) can be used to generate specific T cells for reinfusion. IL-12 is a heterodimeric molecule consisting of two chains of molecular weight 35 (p35) and 40 (p40) kDa respectively. It is produced by macrophages and other APCs (Macatonia et al 1995) and acts on NK and T cells to release primarily IFN-γ. It favours the production of the Th1 subset of CD4+ cells (Kobayashi et al 1989, Manetti et al

1993) and may act antagonistically with IL-4, which favours the Th2 subset of CD4+ cells, and interleukin 10 (IL-10). It also enhances the proliferation of T cells in response to PHA but has little or no effect on its own (Kobayashi et al 1989, Wolf et al 1991, Gately et al 1991). In influenza models in mice, Th1 and Th2 clones have been adoptively transferred and resulted in protection and cytolysis when Th1 clones were used but exacerbation of infection when Th2 clones were used (Graham et al 1994). IL-12 can therefore be used to generate a specific population of Th1 cells which may, when reinfused, provide more help to CD8+ cells and thus augment cytolytic killing. This study aims to address some of these questions and to act as a foundation for developing this form of therapy further.

5.2. Materials and Methods

Animal	Chronic (C)/ Naive (N)	WHV DNA	WHeAg	anti-WHc
W360	C	+	+	+
W414	C	+	+	+
W520	C	+	+	+
W527	C	+	+	+
W507	C	+	+	+
W518	C	+	+	+
W540	C	+	+	+
W556	C	+	+	+
W32	N	0	0	0
W939	N	0	0	0

Table 31. WHV DNA Status of all Animals Employed in IL-12 Assays. (+ present 0 absent)

5.2.1. Effect of Murine and Human IL-2 on the Proliferation and Survival of T cells in Response to PHA

PBLs were obtained from 2 woodchucks,W360 (chronically infected) and W939 (uninfected), as outlined in chapter 2. PBLs were separated and washed as before and the following reactions set up. 1 x 10^5 cells were incubated with 5ug/ml PHA for 4 and 7 days. Murine IL-2 (mIL-2) was kindly supplied by Dr. P. Openshaw, from an X360 cell line secreting IL-2 into the supernatant, at a concentration of 1000U/ml. Human recombinant IL-2 (hIL-2) was supplied at a concentration of 200U/ml (Boehringer Mannheim). Four concentrations of IL-2 were tested - 5, 10, 20 and 50U/ml on each animal for 4 and 7 days with addition of IL-2 on day 1. IL-2 was also added on day 4 and incubated for a further 3 days (7 day maintenance assay). The final volume in the well was 200ul and cells were pulsed and harvested as before. The Kruskall-Wallis test was performed on the data to determine whether differences were significant. p values <0.05 were considered significant.

5.2.2. Effect of Mouse/woodchuck chimaeric and woodchuck IL-12 Molecules on the Proliferation of T cells in the Presence and Absence of PHA or Peptide

IL-12 was made and kindly provided by Dr. A. Ackrill (Hoffman/La Roche) who employed the following methodology- woodchuck spleen cells were stimulated with PHA for two days and then spun and frozen. The 2 genes encoding the 2 subunits of IL-12 were cloned separately from the cell preparation using random primers. After selection of appropriate sequences chinese hamster ovary cells (CHO) were co-

transfected either with the gene encoding the mouse p40 subunit and the gene encoding the woodchuck p35 subunit, so yielding a chimaeric mouse/woodchuck IL-12, or with the genes encoding the woodchuck p40 and p35 subunits. Positive clones were selected using methotrexate and grown in culture medium. The supernatant contained an unquantified amount of IL-12 and was used directly in assays.

Chimaeric IL-12 (cIL-12) consisted of a mouse p40 subunit and woodchuck p35 subunit and woodchuck IL-12 (wIL-12) consisted of both subunits obtained from a woodchuck. 1 x 10^5 cells, obtained from both chronically infected and uninfected animals, were incubated with 20, 50 or 100ul cell supernatant containing IL-12. As controls, medium from untransfected chinese hamster ovary cells was used in addition to the woodchuck p40 homodimer in medium. Cells were also incubated with 5ug/ml PHA and IL-12 together or 5ug/ml peptide and wIL-12 together. All assays were incubated for 4 days and pulsed and harvested as before. The Mann-Whitney U-test and Kruskall-Wallis test was performed on the data to determine whether differences were significant. p values <0.05 were considered significant.

5.2.3. Investigation of the Ability of T cells to Survive in Vivo After Stimulation with Peptide and Subsequent Reinfusion

Pre-bleeds were obtained on three consecutive weeks prior to reinfusion. 6mls of blood was obtained from W507 and W518 and PBLs separated and washed in the usual manner. 5 x 10^6 cells were incubated in 24 well plates (Nunc) with 5ug/ml peptide 38-52 (chapter 3) for 4 days. The final volume in the well was 2mls. Cells

were pulsed with 50uCi ^{3}H-uridine for 6 hours as before and washed in RPMI-1640 medium. Cells were counted and resuspended in 1ml medium before reinfusion back into the same animal via the antecubital vein of the hind leg. At the same time 1 x 10^5 cells were incubated with 5ug/ml peptide 38-52 in a 96-well plate and incubated, pulsed and harvested as before (chapter 3). Blood samples were obtained 4 days after reinfusion and PBLs separated and washed as before. 1 x 10^5 cells were plated out and harvested before counting in order to detect any radioactivity.

5.2.4. Effect of Reinfusion of T cells Stimulated with Peptide Only on the Course of Persistent WHV Infection

W507 and W518 were anaesthetized and bled 4 weeks later and 10mls of blood were obtained. Cells were isolated and incubated with peptide 38-52 as in experiment 5.2.3. After 4 days cells were washed and counted but not pulsed. 26.25 x 10^6 and 14 x 10^6 cells were reinfused back into W507 and W518 respectively. Blood samples were taken before reinfusion for three weeks and biweekly for 2 weeks following reinfusion and used to monitor WHV DNA, WHeAg and GGT levels as outlined in chapter 4.

5.2.5. Effect of Reinfusion of T cells Stimulated with Peptide and wIL-12 on the Course of Persistent WHV Infection

W540 and W556 underwent the same procedure as outlined in section 5.2.4 except with the use of peptide 91-105. Cells and peptide were present in a volume of 1ml to which 1ml wIL-12 was added before incubation for 4 days. Cells were then washed

and counted and 21.25 x 10^6 and 16.5 x 10^6 cells were reinfused back into W540 and W556 respectively. Blood samples were taken three weeks prior to reinfusion and biweekly following reinfusion of cells and used to monitor the markers as mentioned in 5.2.4.

5.3 Results

5.3.1. Effect of murine and human IL-2 on the proliferation and survival of T cells in response to PHA

The optimal concentration of hIL-2 after 4 days in culture using W360 (chronically infected) cells was 20U/ml ($p=0.03$) and that of mIL-2 10U/ml ($p=0.03$) (figure 76). Using lymphocytes from W939 (uninfected) the optimal concentration of both hIL-2 and mIL-2 was 10U/ml (0.03) (figure 77). Mean cpm values obtained with IL-2 were significantly different from mean cpm values obtained with PHA alone.

After 7 days in culture the optimum concentration of both hIL-2 and mIL-2 in both animals was 20U/ml ($p=0.03$ in W360 and $p=0.03$ in W939). The cells stimulated with PHA only had reduced incorporation of Udr in both animals (figures 78 and 79).

When IL-2 was added to the cultures on day 4 using lymphocytes from W360 the optimal concentration of hIL-2 was 20U/ml ($p=0.03$) whereas mIL-2 produced an increased response when used at 50U/ml ($p=0.03$)(figure 80). Using lymphocytes from W939 the optimal stimulation was obtained with 10U/ml hIL-2 ($p=0.05$) and 50U/ml of mIL-2 ($p=0.03$) (figure 81).

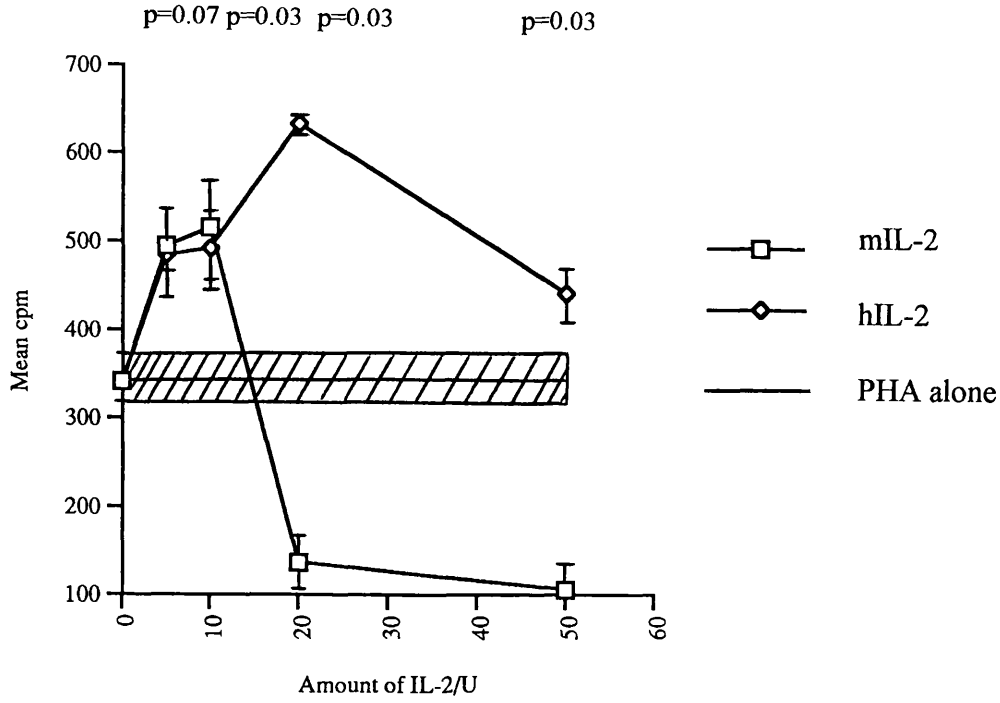


Figure 76. Graph to compare the effect of hIL-2 and mIL-2 on T cell proliferation after 4 days in W360. p values < 0.05 were considered significant (Kruskall-Wallis test). The shaded area indicates the mean cpm obtained using PHA alone ± s.e.m.

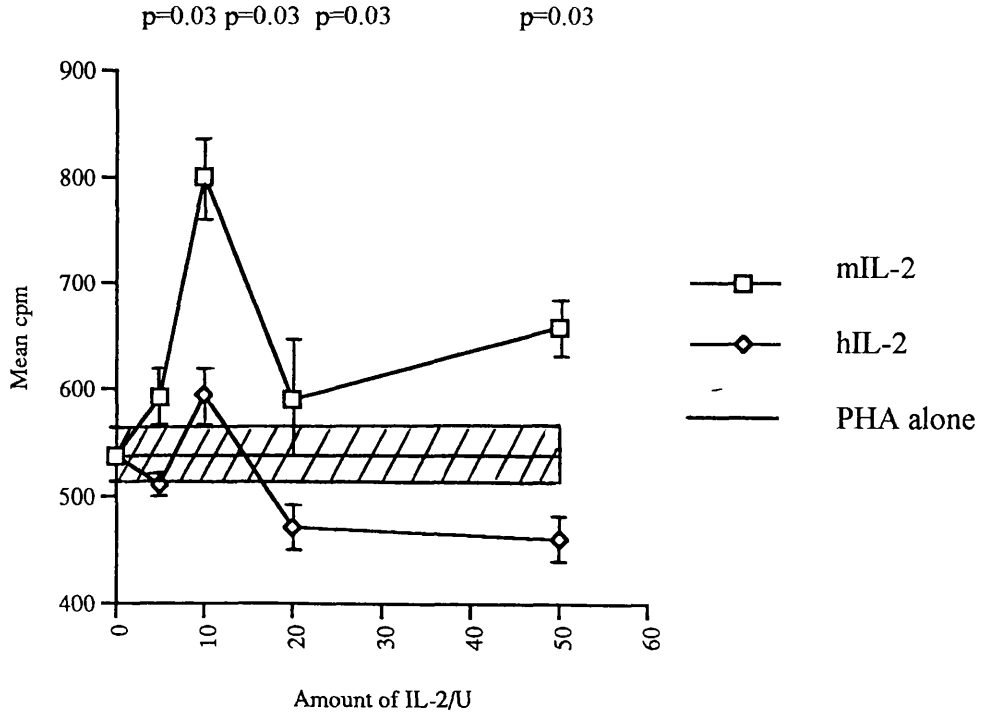


Figure 77. Graph to compare the effect of hIL-2 and mIL-2 on T cell proliferation after 4 days in W939. p values < 0.05 were considered significant (Kruskall-Wallis test). The shaded area indicates the mean cpm obtained using PHA alone ± s.e.m.

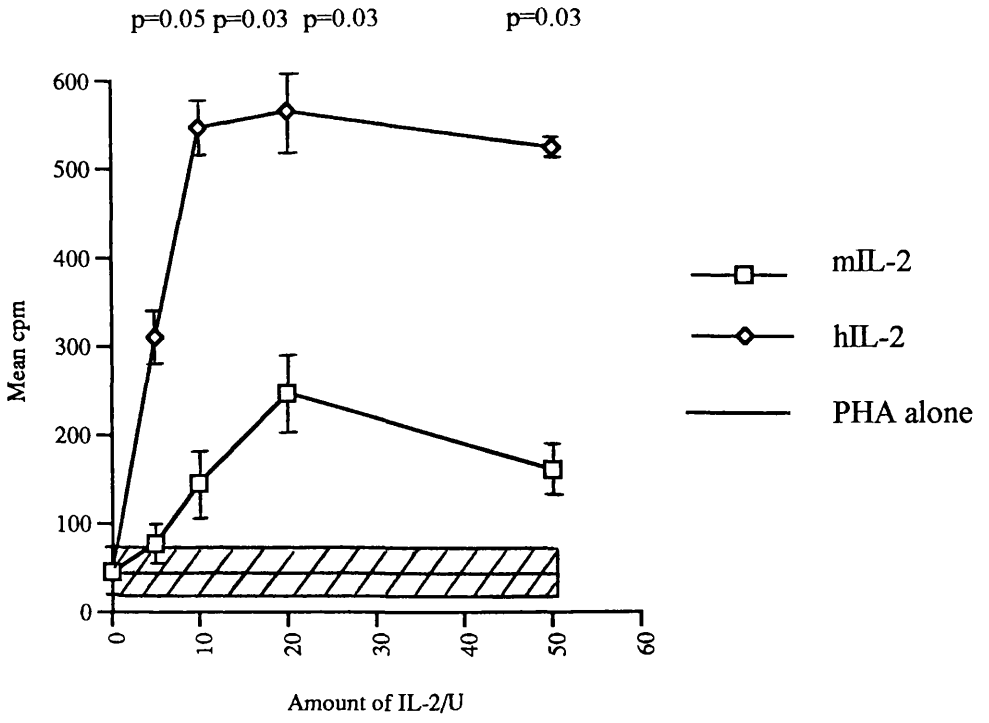


Figure 78. Graph to compare the effect of hIL-2 and mIL-2 on T cell proliferation after 7 days in W360. p values < 0.05 were considered significant (Kruskall-Wallis test). The shaded area indicates the mean cpm obtained using PHA alone ± s.e.m.

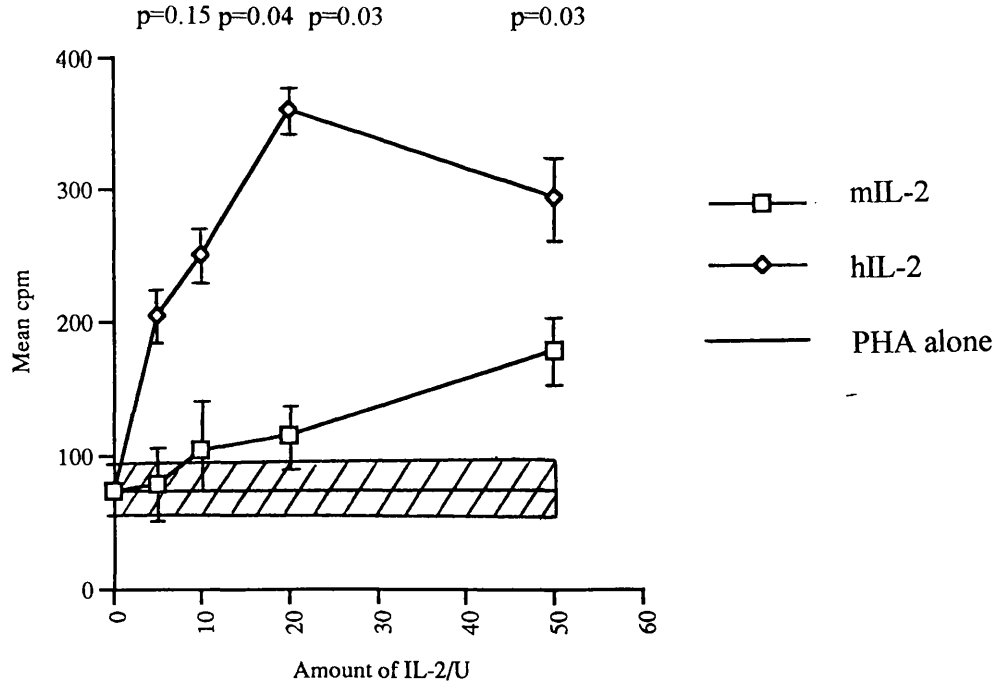


Figure 79. Graph to compare the effect of hIL-2 and mIL-2 on T cell proliferation after 4 days in W939. p values < 0.05 were considered significant (Kruskall-Wallis test). The shaded area indicates the mean cpm obtained using PHA alone ± s.e.m.

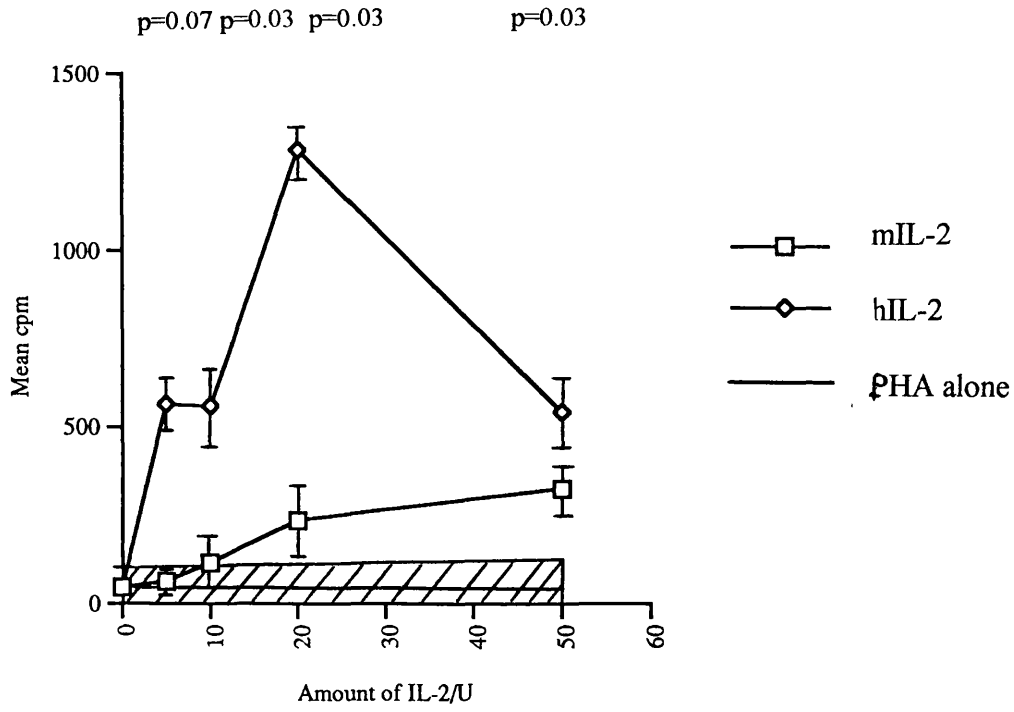


Figure 80. Graph to compare the effect of hIL-2 and mIL-2 on T cell proliferation after 7 days (maintenance) in W360. p values < 0.05 were considered significant (Kruskall-Wallis test). The shaded area indicates the mean cpm obtained using PHA alone ± s.e.m.

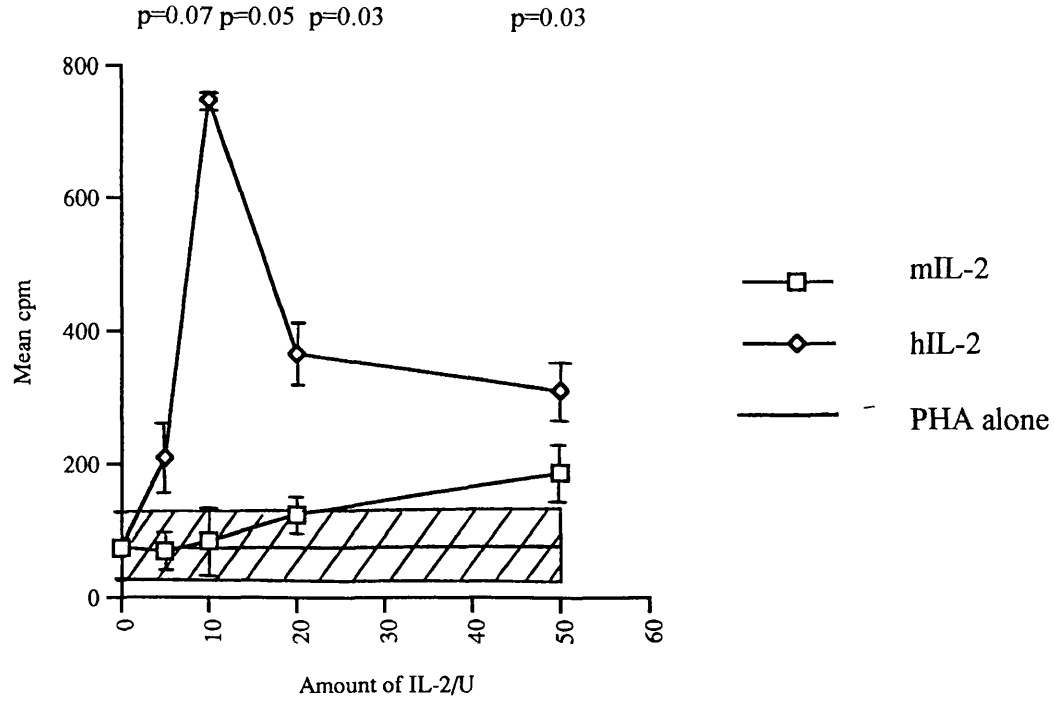


Figure 81. Graph to compare the effect of hIL-2 and mIL-2 on T cell proliferation after 7 days (maintenance) in W939. p values < 0.05 were considered significant (Kruskall-Wallis test). The shaded area indicates the mean cpm obtained using PHA alone ± s.e.m.

5.3.2. Effect of Mouse/woodchuck chimaeric and woodchuck IL-12 Molecules on the Proliferation of T cells in the Presence and Absence of PHA or Peptide

The optimal amount of cIL-12 to boost the proliferative response to PHA in 4 day cultures of lymphocytes from W414 (infected) and W939 (uninfected) was 50ul (figures 82 and 83). There was no significant difference in the response to cIL-12 between W414 and W939 (p values = 0.08). The lymphocytes of W360 (infected) responded optimally to PHA in the presence of 5ul cIL-12 (figure 84) although the mean cpms were not significantly different from PHA alone (p values = 0.08). This amount was then used to examine the effect on the dose response to PHA in this animal (figure 85). There was no significant difference in the response to PHA in the presence of cIL-12 (p value = 0.08); it was noted however, that the response was higher.

Woodchuck lymphocytes from 2 chronically infected animals proliferated when they were incubated in the presence of the p40 homodimer or wIL-12 (figure 86 and 87). There was a significant difference in the responses in W520 (p value = 0.03) and also in W527 using 50 and 100ul of supernatant (p value = 0.03).

The lymphocytes from a third animal (W32) also proliferated in response to the p40 homodimer and wIL-12 (figure 88). There was a significant difference in the response to the supernatants using 50 and a 100ul in the assay (p value = 0.03). However, only wIL-12 increased the proliferative response to PHA and only when 50ul was used (figure 89). There was a significant difference in the response to the supernatants

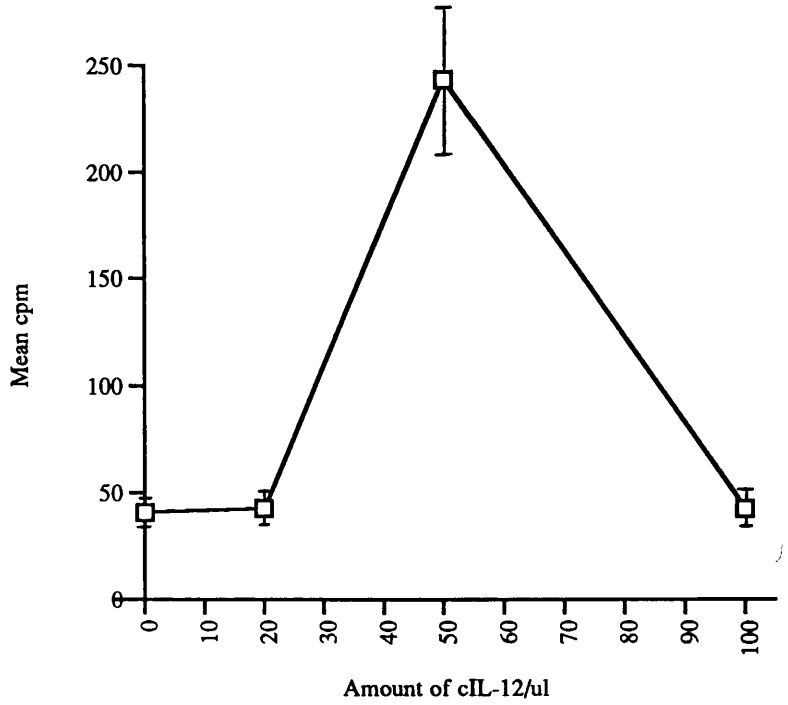


Figure 82. Graph to Illustrate the Effect of cIL-12 on T cell proliferation in W414.

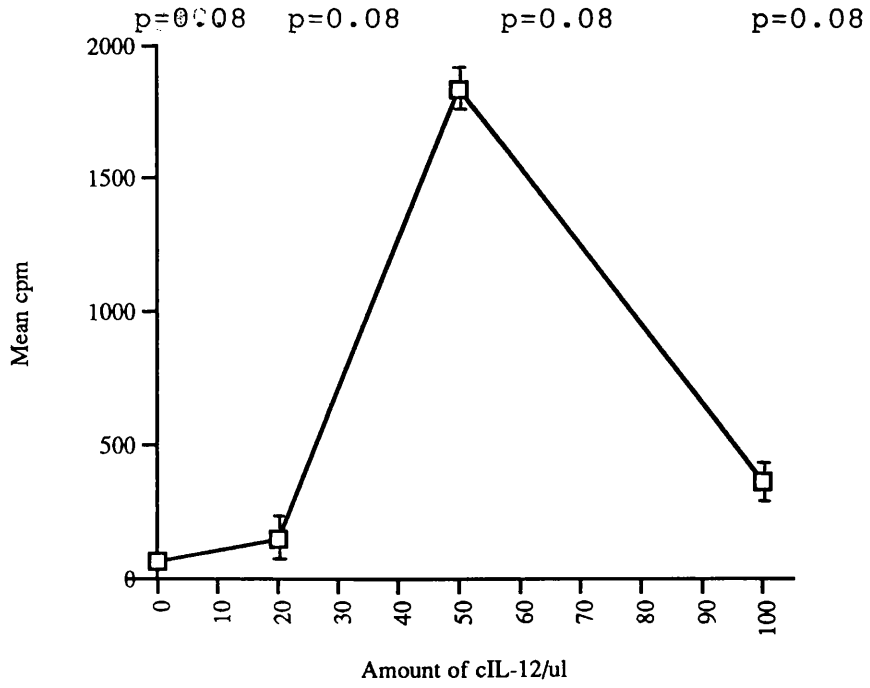


Figure 83. Graph to Illustrate the Effect of cIL-12 on T cell proliferation in W939. Mann-Whitney U-test was used to compare results from figure 82; p values <0.05 were considered significant.

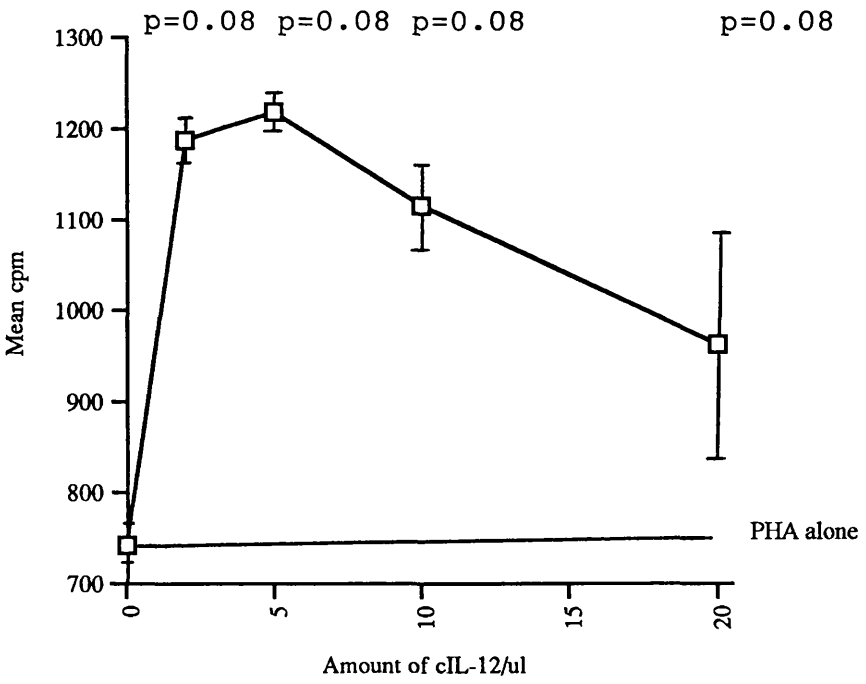


Figure 84. Graph to Illustrate the Effect of Increasing amounts of cIL-12 on the response of T cells to 5ug/ml PHA in W360. p values <0.05 were considered significant (Mann-Whitney U-test)

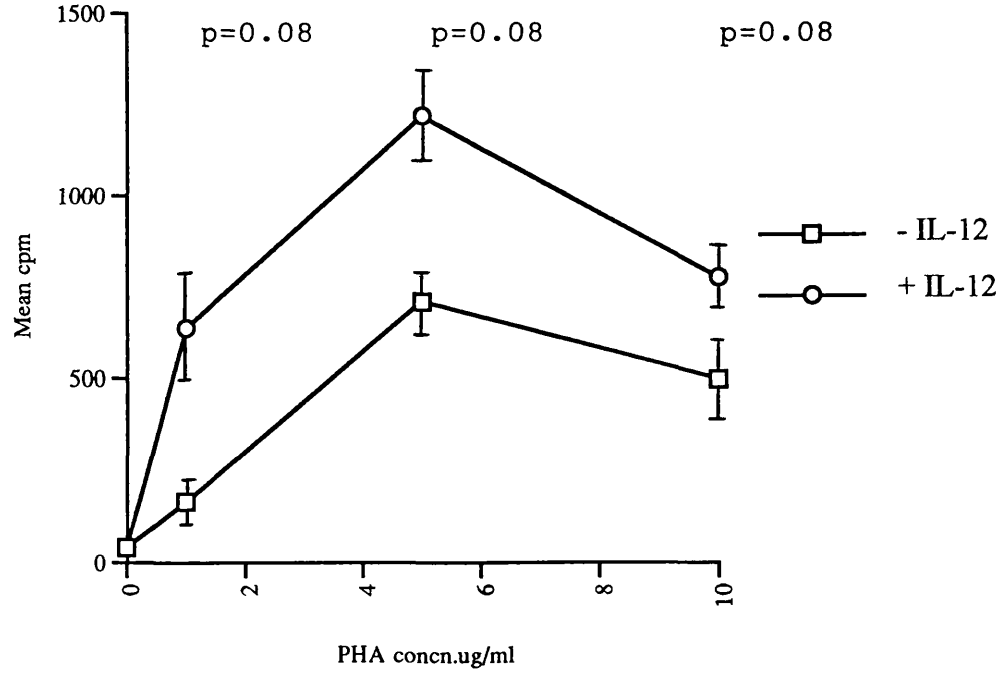


Figure 85. Graph to Illustrate the Effect of 5ul cIL-12 on the Dose-Response of T cells to PHA in W360. p values < 0.05 were considered significant (Mann-Whitney U-test)

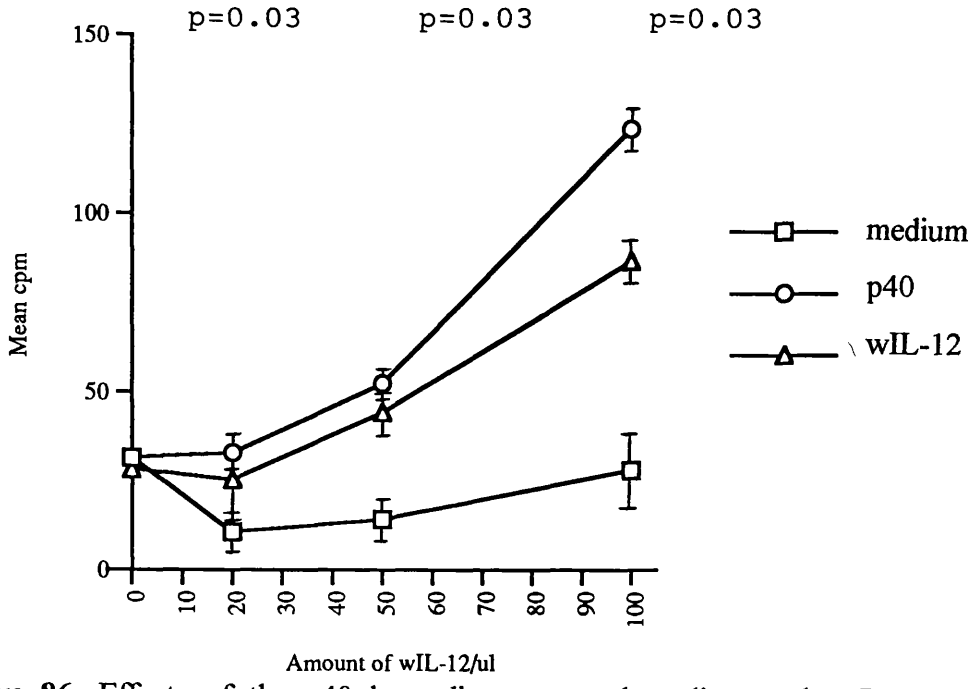


Figure 86. Effects of the p40 homodimer, control medium and wIL-12 on the proliferation of T cells from W520. p values < 0.05 were considered significant (Kruskall-Wallis test)

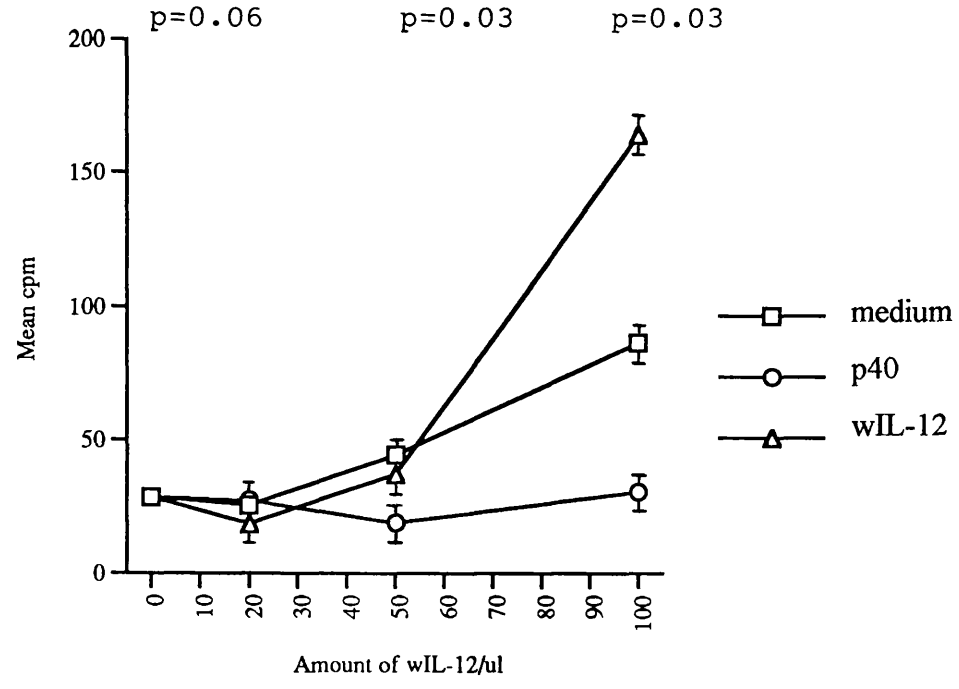


Figure 87. Effects of the p40 homodimer, control medium and wIL-12 on the proliferation of T cells from W527. p values < 0.05 were considered significant (Kruskall-Wallis test)

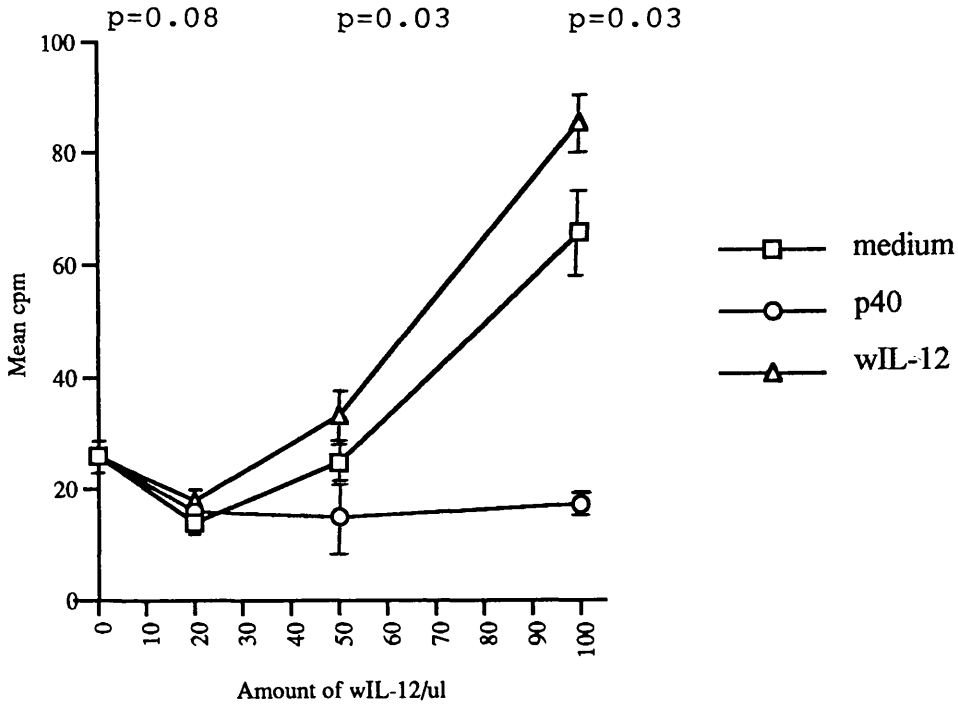


Figure 88. Graph to Illustrate the Effect of the p40 homodimer, control medium and wIL-12, on the Proliferation of T cells from W32. p values < 0.05 were considered significant (Kruskall-Wallis test)

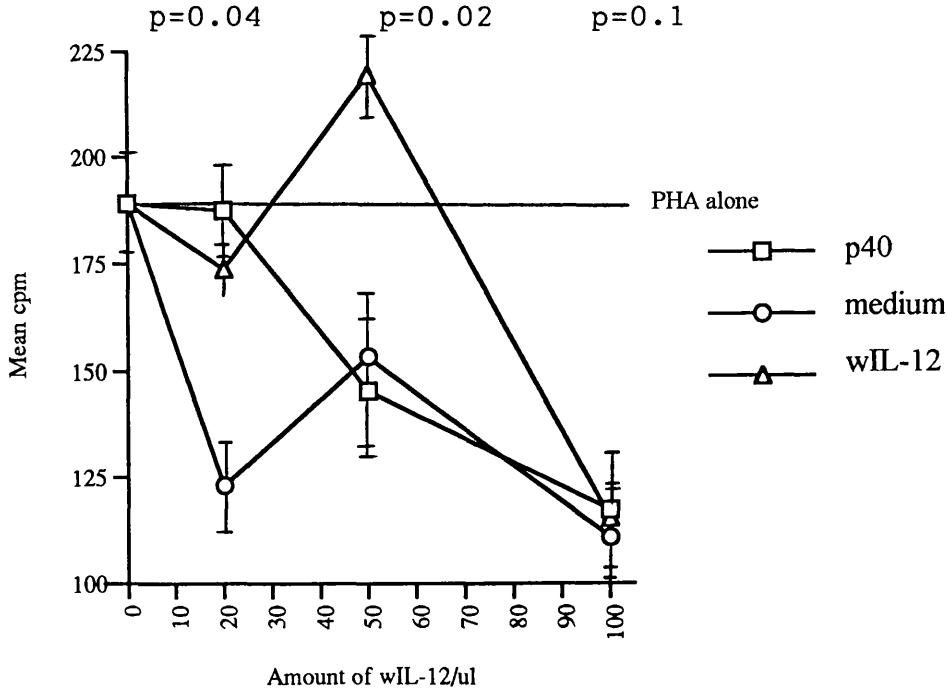


Figure 89. Graph to Illustrate the Effect of the p40 homodimer, control medium and wIL-12, on the Proliferation of T cells from W32 stimulated with 5ug/ml PHA. p values < 0.05 were considered significant (Kruskall-Wallis test)

using 20 and 50ul (p values = 0.04 and 0.02 respectively). However, 100ul wIL-12 produced a higher response when used together with peptides from two different infected animals (figures 90 and 91). Medium alone did not stimulate the cells. The differences in the responses however, were not significant (p value = 0.05)

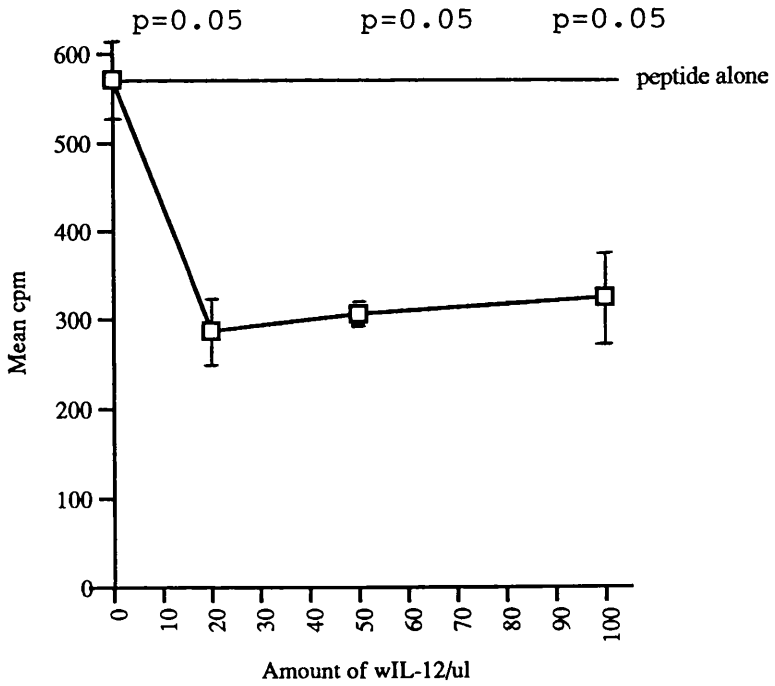


Figure 90. Graph to illustrate the effect of wIL-12 on the proliferation of T cells from W507 stimulated with 5ug/ml peptide 91-105. p values < 0.05 were considered significant (Mann-Whitney U-test)

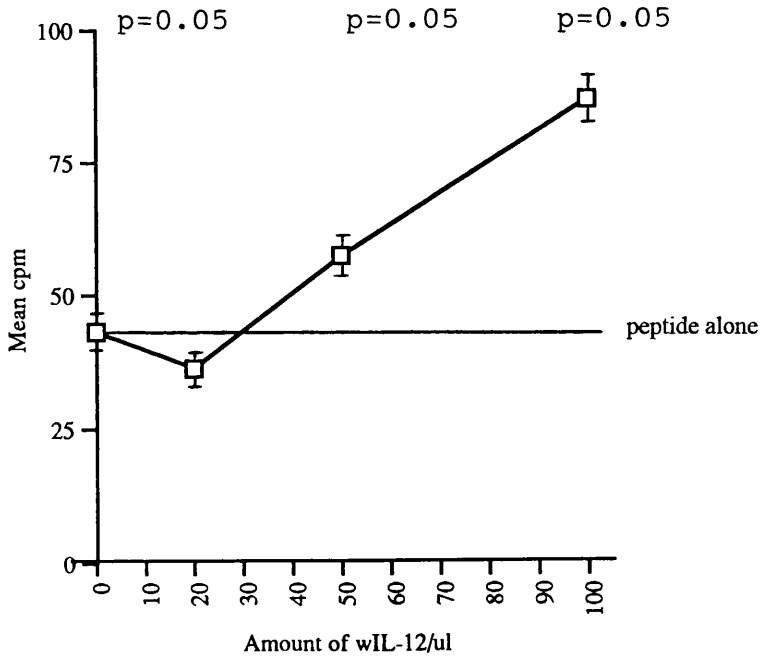


Figure 91. Graph to illustrate the effect of wIL-12 on the proliferation of T cells from W527 stimulated with 5ug/ml peptide 38-52. p values < 0.05 were considered significant (Mann-Whitney U-test)

5.3.3. Investigation of the Ability of T cells to Survive in Vivo After Stimulation with Peptide and Subsequent Reinfusion

19.5 x 10^6 and 15 x 10^6 cells were recovered from 6mls of blood from W507 and W518 respectively. After incubation, pulsing and washing this was reduced to 8.75 x 10^6 and 5 x 10^6 cells respectively. 1 x 10^5 cells from W507 had a mean cpm of 570 and from W518 a mean cpm of 680 prior to reinfusion. No radioactivity could be detected 4 days after reinfusion.

5.3.4. Effect of Reinfusion of T cells Stimulated with Peptide Only on the Course of Persistent WHV Infection

W507 and W518 yielded 30.75 x 10^6 and 26 x 10^6 cells from 8 and 10mls of blood respectively. After incubation and washing this was reduced to 26.25 x 10^6 and 14 x 10^6 cells respectively. W507 showed an increase in WHeAg levels 4 days after infusion and continued to rise for another 8 days before decreasing again (figure 92). WHV DNA levels remained steady 4 days after infusion, then rose and fell for the following 12 days. W518 showed a small increase in WHeAg levels after 4 and 8 days following infusion which then continued to rise before a drop 16 days after infusion (figure 93).

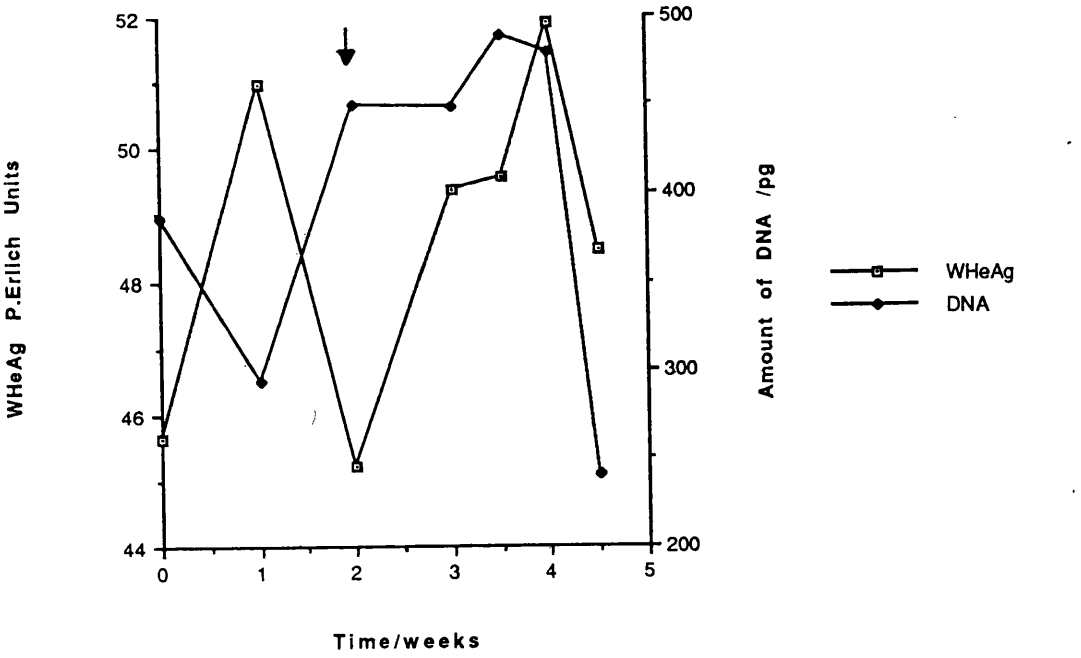


Figure 92. Graph to Illustrate the Effect of Infusion of T lymphocytes Stimulated with Peptide only on the Course of Persistent Infection in W507. Arrow indicates time of infusion.

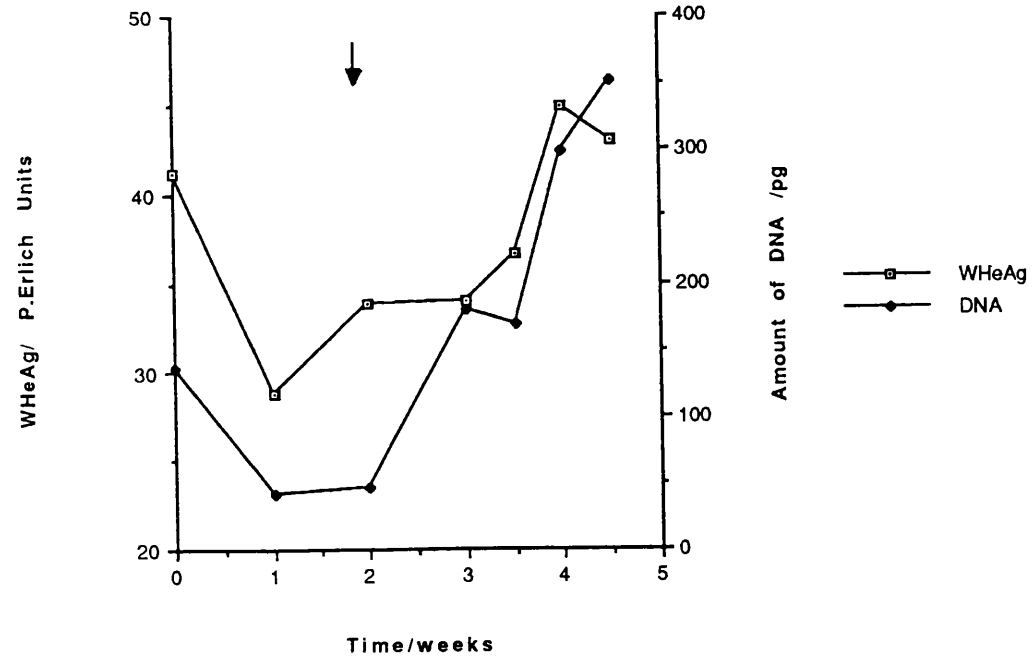


Figure 93. Graph to Illustrate the Effect of Infusion of T lymphocytes Stimulated with peptide only on the Course of Persistent Infection in W518. Arrow indicates time of infusion.

5.3.5. Effect of Reinfusion of T cells Stimulated with Peptide and wIL-12 on the Course of Persistent WHV Infection

W540 and W556 yielded 38.25 x 10^6 and 42.50 x 10^6 cells respectively from 10mls blood each. After incubation and washing this was reduced to 21.25 x 10^6 and 16.50 x 10^6 cells respectively for reinfusion. W540 revealed a steady drop in WHeAg levels after infusion until death which occurred 12 days after infusion (figure 94). WHV DNA levels started to fall before reinfusion and continued to decrease 4 days after infusion, then rose before falling again before death. GGT levels continued to rise to 80.5 IU/L before infusion indicating the development of a tumour (figure 95). After infusion GGT levels fell consistently reaching 35.20 IU/L before death. The cause of death is unknown but the animal did not fully recover from the last dose of anaesthetic. WHeAg and WHV DNA levels remained consistent before reinfusion after which they rose and fluctuated in W556 (figure 96).

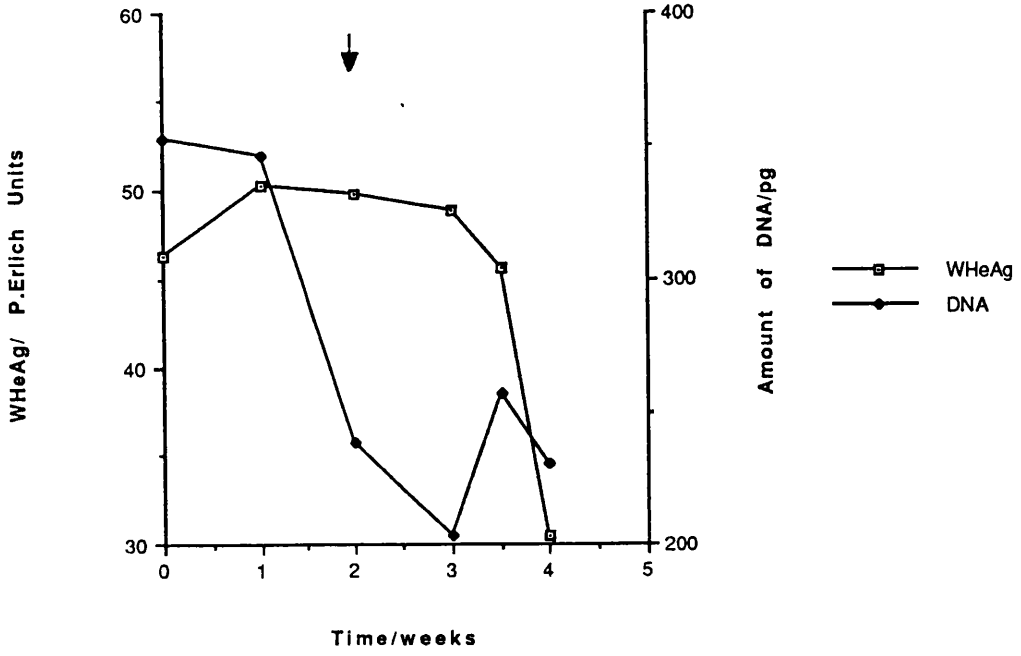


Figure 94. Graph to Illustrate the Effect of Infusion of T lymphocytes Stimulated with Peptide and IL-12 on the Course of Persistent Infection in W540. Arrow indicates time of infusion.

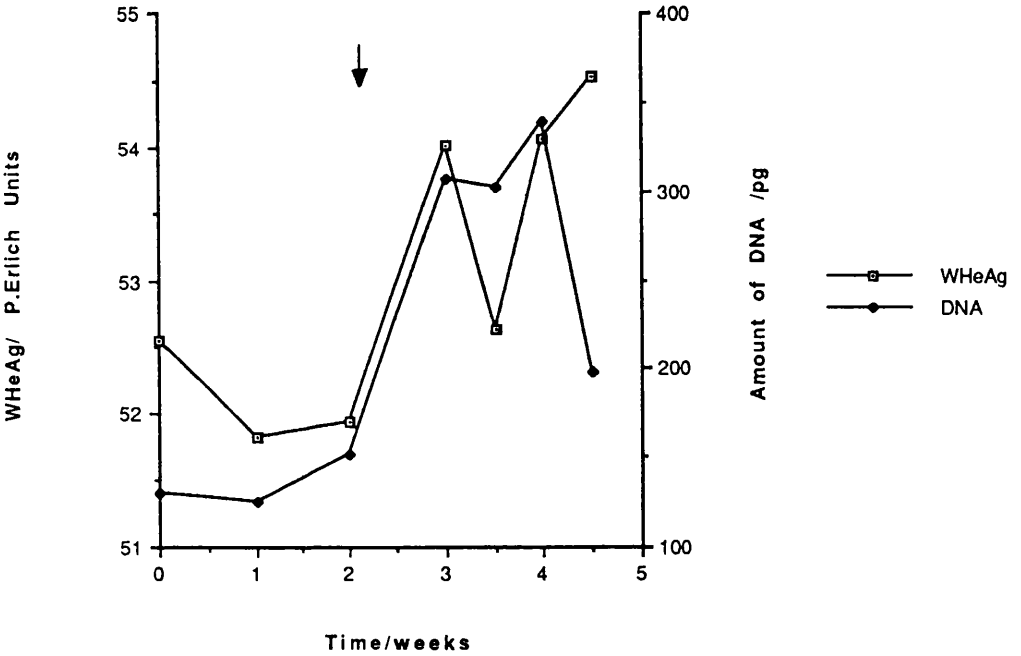


Figure 95. Graph to Illustrate the Effect of Infusion of T lymphocytes Stimulated with Peptide and IL-12 on the Course of persistent Infection in W556. Arrow indicates time of infusion.

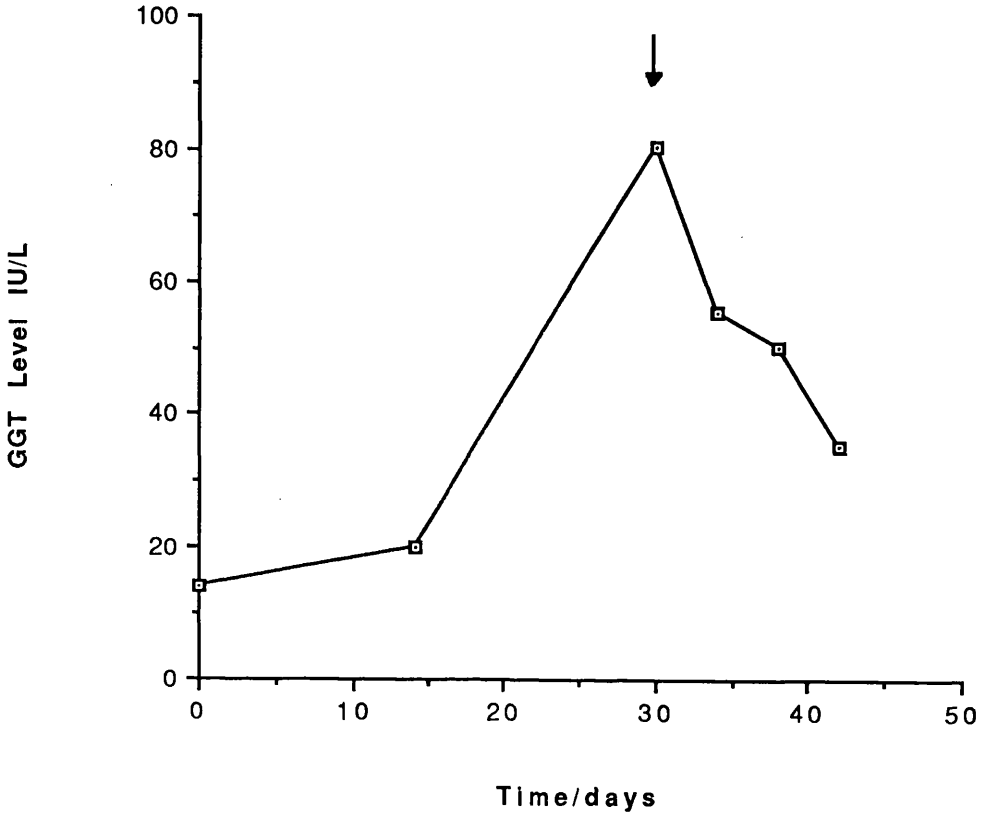


Figure 96. Graph to illustrate the effect of infusion of T cells stimulated with peptide plus IL-12 on GGT levels in W540. Arrow indicates time of infusion.

5.4 Discussion

In order to be able to produce a woodchuck T cell line the response to IL-2, if any, had to be determined. In addition to investigating the long term survival of the cells in vitro we wished to generate a larger number of cells in the short term for reinfusion. Two preparations were available - human IL-2 and murine IL-2, between which there exists 60% amino acid sequence homology. A comparison of their activity was made using cells stimulated with PHA from one infected (W360) and one uninfected (W939) woodchuck. Both species significantly enhanced the stimulation of PBLs by PHA. After 4 days of incubation hIL-2 produced a better response in W360 than mIL-2 at the same concentration, whereas this was reversed in W939. In W360 the disparity is clear at a concentration of 20U/ml but in W939 there are distinct dose response curves for hIL-2 and mIL-2.

After 7 days of incubation cell death was prevented and in this hIL-2 was superior to mIL-2, inducing a strong T cell response in both animals. In the 7 day maintenance assays where IL-2 was added on day 4, hIL-2 also performed better producing a much higher mean cpm value in both animals.

Another interesting point is that in all the assays W360 responds better to hIL-2 than W939. One possible reason for this may be that in the infected animal the immune response may be impaired allowing viraemia to persist. This may be at the T cell level where there is a lack of IL-2 or there is a defect in responsiveness to IL-2. It is clear that when hIL-2 is added after 4 days cell death is prevented and proliferation continues until day 7. This is useful in developing the long term survival of the cells.

This occurs at comparable doses to that used for the generation of human T cell lines (10-20U/ml). More animals are required to confirm these observations.

The use of cIL-12 causes proliferation to occur more efficiently in an uninfected animal than an infected animal. This may be due to animal to animal variation or because the cells do not respond to IL-12. Others have found that cells do not respond well to IL-12 alone (Kobayashi et al 1989, Wolf et al 1991) but when coupled with PHA a significant change in the response is visible. This is confirmed by this study which shows that both woodchuck and chimaeric IL-12 molecules increased the proliferation of PBLs in response to PHA. Therefore one possible therapeutic strategy for administering IL-12 in vivo to persistently infected animals would be to co-administer protein or peptide.

There were significant differences in the response to wIL-12, medium alone and the p40 homodimer in W520, W527 and W32. Comparative volumes of IL-12 were used and their effectiveness in cultures suggest that there is less wIL-12 secreted than cIL-12.

Both molecules are produced by cotransfection of the p40 and p35 subunit RNAs into chinese hamster ovary cells (CHO) and induced to secrete IL-12 into the medium. The level of expression may vary and it may be difficult to assess levels of secretion other than by a bioassay using T cells. It is possible that the difference in response between cIL-12 and wIL-12 is due to the amount present rather than the difference in the molecules. However, we would expect wIL-12 to perform better than the

chimaeric molecule. A more accurate method of comparison is not possible at this stage because there is no available assay to quantify the IL-12 in the supernatant.

The amount of blood that can be obtained from woodchucks varies from animal to animal. 6mls were the maximum obtained from W507 and W518. These cells were used to investigate the ability of the cells to survive in vivo and were labelled with uridine (Udr). We wished to avoid sacrificing the animal and therefore analysis of the organs was not possible so BrdU was not employed. Udr was the only viable alternative and was known to effectively label the cells (chapter 2). No label could be detected 4 days after reinfusion in the peripheral blood. However, this does not mean that the cells did not survive. They may have been sequestered in the liver and the only means of determining this would have been to sacrifice the animal. The results of this experiment were therefore inconclusive.

After reinfusion of peptide specific T cells using a peptide known to stimulate a good proliferative response in those animals there did not appear to be any visible side effects although bi-weekly anaesthetic and bleeding appeared to cause a loss of weight in W518. It was thought that by infusing a predominantly CD4+ population more help would be provided to the CD8+ cells which might have resulted in an increase in cytolytic activity. The effect of this augmentation could be monitored by measuring WHV DNA and WHeAg levels.

It was found that in W507 there was no effect on the WHeAg levels and the fluctuation observed after infusion represents normal variation. The WHV DNA levels

however, appeared to remain steady 4 days after infusion. During the following 8 days WHV DNA levels dropped which may indicate a latent effect of the cells which was confirmed by the drop in WHeAg levels during the last 4 days of the experiment.

W518 did not appear to benefit from the infusion of cells as both WHV DNA and WHeAg levels rose following infusion. The fluctuations observed may represent normal variation in both markers for this animal.

Reinfusion of cells stimulated with peptide and IL-12 appeared to be well tolerated. Both W540 and W556 were easier to bleed and consequently more blood was obtained from them. A similar number of cells to the previous experiment were reinfused.

W540, upon infusion of cells treated with IL-12 and peptide, revealed a drop in both WHeAg and WHV DNA levels 4 days after infusion, indicating a cessation in viral replication, which continued as evidenced by the consistent drop in WHeAg levels. The DNA levels fluctuated before a drop before death. This animal did not recover from the last anaesthetic administered which may be due to impaired liver function. The animal had a developing tumour and GGT levels reached 80 IU/L before infusion which then continued to drop until death. It is clear that the cells had an effect on tumour growth but its role in causing the death of the animal is uncertain. It is more likely that failure to metabolise the anaesthetic arose due to the presence of a tumour impairing liver function.

W556, also treated with cells stimulated with peptide and IL-12, did not have a tumour when infusion occurred and therefore its GGT levels remained normal throughout. There also appeared to be no effect on WHV DNA and WHeAg levels which rose sharply 4 days after infusion and continued to fluctuate thereafter. This is likely to be normal variation in levels in this animal.

Chapter 6

Summary

Although it has been known for some time that the woodchuck harbours a virus sharing 60% nucleotide sequence homology with HBV, the animal has mainly been used for studies on HCC, principally the involvement of DNA integration in the development of carcinoma. The reason why it has not been exploited as an animal model of HBV infection lies in the lack of knowledge regarding the immunological parameters of disease. Whilst much information has been gathered on the immune response in HBV infection, little is known about this system in WHV infection in woodchucks. There is a need for an animal model of HBV infection which can be used for testing new treatments for HBV which include anti-virals and involve the amplification of either the cellular or humoral arm of the immune response. The projects' primary focus was to define the immunological parameters of the woodchuck and to determine whether it was a viable animal model for the study of HBV immunopathogenesis.

The cellular arm of the immune response was targeted because the T cells are thought to play a pivotal role in the immunopathogenesis of hepatitis B. Attempts to differentiate between the CD4+ and CD8+ populations by staining with monoclonal antibodies raised against rat, mouse and human CD4 and CD8 molecules were unsuccessful as the antibodies did not crossreact with woodchuck CD molecules.

Previous attempts to conduct proliferation assays employing woodchuck PBLs foundered due to the difficulties encountered in determining culture conditions for growth. Once the PBLs had been isolated on Ficoll-Paque, it was found that

conventional culture conditions, employed successfully to culture human PBLs, were unsuitable for culturing woodchuck lymphocytes. The cells were only supported by a heavily amino acid enriched medium supplemented with woodchuck serum. Interestingly, woodchucks deviated from other rodents in their inability to incorporate thymidine. This was found to be due to the low level of thymidine kinase in the cells and is not unusual in this group of sciurid rodents (Adelstein et al 1965). In addition they differed from other animal cells in the kinetics of their response, in particular a 4 day incubation period resulted in peak proliferation; by 7 days most of the cells died. Addition of hIL-2 or mIL-2 prolonged their survival when used in conjunction with PHA. The use of new culture conditions proved to be a significant breakthrough in the work as a system had now been devised which reliably assessed T cell proliferative responses. This allowed us to investigate the proliferative T cell response in the pathogenesis of the infection and also to assess the effects of various treatments on T cell responses and their effect on persistent viraemia.

In humans it has been noted that CD4 T cells are sensitised to HBcAg and that the presence of these cells is associated with recovery from HBV infection (Ferrari et al 1991). We investigated the T cell response to WHcAg in the chronically infected woodchuck as manipulation of this response may increase its effectiveness and resolve infection. Peptides from the WHcAg were constructed and immunodominant epitopes found; these varied in different animals. This was the largest study undertaken in this thesis employing 16 animals in total. From this work a clear picture of immunodominant epitopes emerged, as has been found in humans (Ferrari et al 1991, Jung et al 1995).

At the same time new data was emerging from work on patients suggesting that an acutely infected patient had a variable response depending on which time point lymphocytes were tested (Jung et al 1995). Attempts to investigate this in the woodchuck were hampered by resistance to infection: several inocula were injected into W939 but did not produce an infection. W939 remained anti-WHc negative throughout. The age of the animal may have been the significant factor as new born woodchucks have been found to be more easily infected than adult animals (Popper et al 1987). It may be more likely for animals to acquire the infection at birth or in utero when the immune system is not fully developed. We were unable to compare the response of an acutely infected animal with a chronically infected animal but this would be of interest to further characterise the similarity with the course of HBV infection.

Monitoring WHV DNA levels for the development of an acute infection is labour intensive. We wished to use a more convenient marker of infection and therefore investigated the antigenic crossreactivity exhibited between WHV and HBV antigens, in particular WHeAg and WHsAg. WHeAg was found to crossreact with HBeAg and this allowed us to employ a commercial assay kit to assess its level in infected animals. We observed that each animal who was WHV DNA positive was also positive for WHeAg. HBeAg negative mutants are known to exist in HBV infection which allow patients to be WHV DNA positive and HBeAg negative. To date no evidence has been found to support the existence of WHeAg negative mutants in WHV infected woodchucks. We therefore used the presence of WHeAg as an additional marker of

infection, which confirmed the WHV DNA status of the animals and could be measured using a quick and convenient method. Together 4 markers could be monitored during treatment - WHV DNA, WHeAg, T cell proliferative responses and GGT levels. This provided us with a sound basis for assessing the effect of treatments on the immune system and viraemia.

As outlined in the introduction, therapies focused on the different stages of the cellular immune response. The initiation step is the recognition of antigen which is then processed by the APC. The second step of the immune response, the interaction between APCs and T cells, was the target of Tucaresol, an immunopotentiating drug tested prior to trials in patients at three different doses. HBeAg was administered with the drug as it shares some sequence homology with WHeAg but is sufficiently different to break tolerance to WHeAg in unresponsive animals. The clinically relevant response was the eradication of WHV DNA which was not observed in any of the animals except W405. A large degree of variation was observed in WHV DNA, WHeAg levels and T cell responses over a peroid of time. This drug was also found to have potent side effects which resulted in abscess formation leading to secondary infections and on two occasions death. I.M. injection resulted in regression of tumour growth which was unexpected. Oral administration could not be considered due to the difficulties in handling the animals and therefore it was concluded that this drug was too toxic for further use in these animals. However, in humans oral administration is the preferred route.

MPC-866, a synthetic oligomer which binds to double stranded DNA had no anti-viral effect but again appeared to inhibit the growth of tumours in two animals. It may do this by binding to integrated viral DNA sequences involved in cis-activation of cellular proto-oncogenes or by binding to episomal ccc DNA to stop transcription of viral genes such as the WHV x gene which may be involved in transactivation of cellular proto-oncogenes. In addition the drug may, by binding to viral genes in ccc DNA, inhibit viral replication and function as an anti-viral agent. Alternatively it may bind to the region coding for the reverse transcriptase enzyme which would prevent viral replication and also arrest tumour development. Both animals showed no change in WHV DNA, indicating no significant anti-viral activity, but did show a drop in GGT levels at the same time point after drug administration in both animals and a recovery after administration was stopped. The temporal similarity of this effect in both animals suggests an effect of the drug on tumour growth. Whilst GGT is a non-specific marker all animals with an elevated level of GGT possessed a tumour and GGT was used as a reliable marker of HCC development. The results of these two studies suggests that the woodchuck may not be the most suitable animal model of HBV infection. The large degree of variation observed in the parameters monitored makes small changes, possibly due to drug effects, difficult to interpret. The only possible result which would yield clinically useful information is the eradication of WHV DNA and WHeAg.

T cell adoptive transfer addresses the third stage of the immune response - T cell help. T cell adoptive transfer of peptide-specific CD4+ cells is thought to increase the amount of help provided to the CD8+ cells which would result in lysis of infected

hepatocytes expressing WHcAg. We attempted this in two chronically infected animals who showed strong T cell proliferative responses to WHcAg peptides. However, the eradication of WHV DNA or WHeAg from the serum was not observed. Two animals were also infused with CD4 T cells stimulated with peptide and IL-12. A drop in WHV DNA and WHeAg was observed in one animal who had developed a tumour. No change was observed in the other animal who had normal GGT levels. Experiments of this kind have not been attempted in animals of this size and therefore no previous information was available on which to base the dose of cells administered. However, the dosage of cells used was well tolerated and it provides a useful foundation on which to build other experiments such as regular T cell infusion using other cytokines and antigens. More information is however, required about the physiology and metabolism of the woodchuck in order to improve studies of this kind.

IL-12 is a new molecule found to have a potent influence on the development of Th1 cells. It has been postulated (Milich et al 1995) that the balance between the Th1 and Th2 cells is responsible for the development of acute or chronic disease. By tipping the balance of cells in favour of Th1 cells the course of infection could be reversed to resolve persistent infection. IL-12 was found to be effective in vitro on woodchuck PBLs with and without PHA. Although IL-12 was obtained from an unpurified supernatant containing an unquantified amount of protein this work suggests that it may have some effect when used as a treatment in vivo. This is a promising form of therapy which allows the idea of regular reinfusion to remain a feasible proposition.

In conclusion, immunological parameters of the woodchuck system have been identified which has increased the usefulness of this model in testing the safety and efficacy of drug treatments for HBV infection; some early attempts to examine the effects of manipulation of the humoral and cellular immune response, both in vitro and in vivo, have been described. However, from the work conducted so far on this model it is clear that more animals need to be used in pre-clinical trials of drugs in order to obtain meaningful data. Large fluctuations were observed in DNA and WHeAg levels and the most clinically useful endpoint is the eradication of these markers. The elucidation of more information on the haematology and physiology of the woodchuck would improve the design of drug studies and make results easier to interpret. Furthermore, there are a number of uncontrollable factors which can affect the outcome of a study e.g. a developing tumour, period of time the virus has been harboured in wild caught animals, a lack of thymidine kinase, which would determine the usefulness of testing drugs which target this enzyme e.g. acyclovir. As a result of their ferocity, handling of the animals and routes of administration of the drugs are restricted and work would have to be conducted within these constraints. Based on the results of the work conducted in this thesis my tentative conclusion is that the usefulness of this model is limited.

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APPENDIX I

Solutions and Buffers

20X SSC	3M NaCl 0.3M tri-sodium citrate Adjusted to pH 7.0 with NaOH
100X Denhardts solution	2% (V/V) bovine serum albumin (BSA) 2% (W/V) Ficoll 2% (W/V) polyvinylpyrrolidone (PVP)
Phenol/Chloroform 50:50 (W/V)	100g ultra pure phenol 100ml chloroform
50X TE buffer	500mM Tris-HCl pH 7.4 50mM EDTA
50X Tris Acetate (TAE) buffer	2M Tris-acetate 0.1M EDTA
50X Tris-borate (TBE) buffer	4.45M Tris-borate 0.4M EDTA

ALKALINE LYSIS BUFFERS

Solution I	50mM glucose (sterile filtered)
	50mM Tris-HCl pH 8.0
	10mM EDTA pH 8.0
Solution II	1% SDS in 0.2M NaOH (freshly prepared)
Solution III	5M potassium acetate
Triton-X lysis buffer	2% (W/V) Triton-X 100
	72.5mM EDTA
	50mM Tris-HCl pH 8.0

MEDIA FOR CULTURING BACTERIA

L-Broth	1% (W/V) Bacto-tryptone
	1% (W/V) NaCl
	0.5% (W/V) Bacto-yeast
L-Agar	5% bacto-agar in L-broth

MEDIA FOR CULTURING CELLS

Clicks medium	1X Hanks BSS
	2X Essential Amino acids
	5X Non-essential amino acids
	0.1M NaOH
	2.5X Nucleic acid precursors
	2X Vitamins
	2.5X Pyruvate
	2X Glutamine
	25 units penicillin/streptomycin

Phosphate buffered	0.14M NaCl
saline (PBS) 'A'	7mM KCl
Dulbecco	1.5mM KH_2PO_4
	8.1mM Na_2HPO_4

DOT BLOT ANALYSIS SOLUTIONS

2X Summers solution	0.5% Tris
	1.75% NaCl
	0.82% Na_4EDTA
	Adjust pH to 8.0 with concn. Hcl

Pre-hybridisation mix	10ml 20X Denhardt's solution
	16ul 25% SDS
	10ml sterile distilled water
	Stored at -20°C after use.

Hybridisation mix	2.5ml 20X SSC solution
	1.25ml 20X Denhardt's solution
	20ul 25% SDS
	5ml 100% formamide
	1ml sterile distilled water

APPENDIX II

A.

PHA Concn.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	32	101	151	41	96	84	48
1	60	23	21	36	19	32	17
5	61	88	53	79	97	76	18
10	41	117	117	164	122	106	45

B.

PHA Concn.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W31	W34	W32	Mean cpm	S.E.M.
0	1237	1031	816	133	113	666	517
1	859	963	673	206	158	572	371
5	539	867	1515	90	193	640	576
10	323	134	920	42	171	318	351

Table 1. Mean counts per minute (cpm) and standard error of the means (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen PHA. PBLs were cultured in RPMI medium and supplemented with 10% FCS for 3 days and pulsed with Tdr.

A.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	65	1542	151	41	737	507	644
1	133	1713	156	73	779	570	699
5	42	558	191	106	671	314	282
10	136	652	252	136	871	409	334

B.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W31	W34	W32	Mean cpm	S.E.M.
0	1826	1237	245	133	35	695	794
1	757	859	40	48	12	343	426
5	1563	539	41	114	16	454	654
10	1739	516	43	102	32	486	727

Table 2. Mean counts per minute (cpm) and standard error of the means (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in RPMI medium and supplemented with 10% FCS for 3 days and pulsed with Tdr.

A.

LPS Concn.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	65	1542	151	41	737	507	644
1	25	983	43	11	462	305	423
5	51	979	105	29	597	352	420
10	133	1043	203	80	812	454	441

B.

LPS Concn.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W31	W34	W32	Mean cpm	S.E.M.
0	1826	1237	245	133	35	695	794
1	767	1103	35	206	7	423	487
5	793	369	41	90	9	260	329
10	860	603	74	51	24	322	384

Table 3. Mean counts per minute (cpm) and standard error of the means (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen LPS. PBLs were cultured in RPMI medium and supplemented with 10% FCS for 3 days and pulsed with Tdr.

A.

PHA Concn.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	190	609	369	140	224	306	189
1	402	1072	760	302	493	606	311
5	398	1184	892	312	904	738	369
10	512	1371	707	603	1213	881	385

B.

PHA Concn.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W31	W34	W32	Mean cpm	S.E.M.
0	237	137	816	133	865	437	370
1	561	458	673	206	529	485	174
5	720	593	1515	90	413	666	530
10	698	571	1786	93	321	694	653

Table 4. Mean counts per minute (cpm) and standard error of the means (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen PHA. PBLs were cultured in RPMI medium and supplemented with 10% FCS for 4 days and pulsed with Udr.

A.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	190	308	135	140	224	199	71
1	583	868	758	872	482	712	174
5	785	1026	926	1040	880	931	105
10	599	1004	630	789	1003	805	195

B.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W31	W34	W32	Mean cpm	S.E.M.
0	814	137	816	133	865	553	381
1	1509	357	1515	199	469	809	648
5	1628	655	1828	74	524	942	752
10	860	602	1108	64	890	705	400

Table 5. Mean counts per minute (cpm) and standard error of the means (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in RPMI medium and supplemented with 10% FCS for 4 days and pulsed with Udr.

A.

LPS Concn.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	190	772	369	140	224	339	256
1	201	514	440	123	246	305	165
5	189	620	378	145	218	310	194
10	183	403	324	102	194	241	120

B.

LPS Concn.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W31	W34	W32	Mean cpm	S.E.M.
0	865	432	147	760	1020	644	352
1	469	139	187	616	1008	483	353
5	524	114	184	773	1028	524	387
10	613	107	185	673	449	405	252

Table 6. Mean counts per minute (cpm) and standard error of the means (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen LPS. PBLs were cultured in RPMI medium and supplemented with 10% FCS for 4 days and pulsed with Udr.

A.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	90	148	198	232	247	183	64
1	211	276	622	358	341	361	156
5	243	398	748	636	836	572	246
10	200	414	36	33	415	219	190

B.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W867	W876	W32	Mean cpm	S.E.M.
0	229	962	1072	1695	1120	1015	523
1	362	816	1101	1324	1126	945	373
5	318	973	1232	1562	1261	1069	468
10	398	1043	1131	1684	982	1047	457

Table 7. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in RPMI medium and supplemented with 10%mouse serum for 3 days and pulsed with Tdr.

A.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	212	428	306	241	512	339	127
1	257	488	356	232	567	380	145
5	234	568	398	219	609	405	181
10	224	683	416	198	634	431	224

B.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W867	W876	W32	Mean cpm	S.E.M.
0	229	962	1072	1695	1120	1015	523
1	341	893	703	601	1492	806	432
5	288	672	597	576	626	551	151
10	304	324	649	463	750	498	197

Table 8. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in RPMI medium and supplemented with 10% rat serum for 3 days and pulsed with Tdr.

A.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	668	807	840	986	1231	906	213
1	598	852	918	890	1104	872	181
5	683	1089	1140	1004	1307	1044	230
10	696	1054	768	1009	1462	997	301

B.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W867	W876	W32	Mean cpm	S.E.M.
0	903	1567	41	56	682	650	638
1	868	1474	79	70	716	641	589
5	758	1231	111	78	642	564	482
10	796	1068	84	109	431	497	430

Table 9. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in RPMI medium and supplemented with 10% sheep serum for 3 days and pulsed with Tdr.

A.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	1621	1003	829	632	604	937	414
1	902	980	925	741	334	776	263
5	1321	1172	1392	800	469	1031	388
10	1523	1759	855	864	513	1102	518

B.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W867	W876	W32	Mean cpm	S.E.M.
0	972	861	242	323	481	576	324
1	852	918	334	233	276	522	333
5	1089	1140	363	154	398	629	453
10	1054	768	274	78	414	517	391

Table 10. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in RPMI medium and supplemented with 10% rabbit serum for 3 days and pulsed with Tdr.

A.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	305	284	562	893	23	465	345
1	433	610	1437	1034	34	709	542
5	624	548	2323	2697	27	1244	1185
10	1559	675	3002	3921	29	1837	1611

B.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W867	W876	W32	Mean cpm	S.E.M.
0	1763	1232	987	1170	1221	1274	290
1	1861	1240	1003	1305	1462	1374	318
5	2130	1368	1327	1623	1617	1613	319
10	1913	1412	1470	1821	1938	1711	251

Table 11. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in Clicks medium and supplemented with 0.5% mouse serum for 4 days and pulsed with Tdr.

A.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	1753	1542	562	893	23	954	708
1	1071	1808	431	763	30	820	674
5	1379	1033	574	885	26	779	511
10	2697	762	600	972	27	1011	1005

B.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W867	W876	W32	Mean cpm	S.E.M.
0	1763	1232	987	1170	1221	1274	290
1	1423	902	862	1141	1011	1068	225
5	1516	1204	993	1301	1196	1242	189
10	1719	1468	1012	1360	1246	1361	262

Table 12. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in Clicks medium and supplemented with 0.5% rat serum for 4 days and pulsed with Tdr.

A.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	2697	2431	462	510	1346	1489	1047
1	2090	1850	396	486	1152	1194	770
5	2841	1503	412	500	1160	1283	982
10	2038	1278	410	512	1216	1090	660

B.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W867	W876	W32	Mean cpm	S.E.M.
0	1763	1232	987	1170	1221	1197	317
1	1761	1103	851	947	1009	1134	362
5	1821	1263	853	1156	1263	1271	350
10	1800	1302	967	1132	1242	1288	312

Table 13. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in Clicks medium and supplemented with 0.5% sheep serum for 4 days and pulsed with Tdr.

A.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	1836	1402	462	510	1346	111	601
1	2043	1605	387	399	987	1084	734
5	1766	1003	470	550	1340	1025	543
10	821	1018	478	556	1412	857	377

B.

ConA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W17	W30	W867	W876	W32	Mean cpm	S.E.M.
0	1763	1232	987	1170	1221	1274	290
1	962	821	673	864	766	817	107
5	1148	967	824	927	985	970	117
10	1463	1189	975	1176	1341	1228	184

Table 14. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in Clicks medium and supplemented with 0.5% rabbit serum for 4 days and pulsed with Tdr.

A.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	772	788	428	424	369	561	199
1	1350	1209	645	1986	760	1190	534
5	1536	1119	819	2762	892	1425	797
10	2624	1385	646	4243	707	1921	1523

B.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W30	W867	W876	W938	W939	Mean cpm	S.E.M.
0	167	206	132	128	73	141	49
1	861	230	286	935	155	493	373
5	1253	2567	136	1069	79	1021	1014
10	1793	2393	109	1631	101	1205	1043

Table 15. Mean counts per minute (cpm) and standard error of the means (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in Clicks medium and supplemented with 10% woodchuck serum for 4 days and pulsed with Udr.

A.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	305	44	56	70	70	109	110
1	610	182	70	155	250	253	209
5	548	255	78	179	196	251	177
10	675	139	109	104	107	227	250

B.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W30	W867	W876	W938	W939	Mean cpm	S.E.M.
0	42	80	61	71	53	61	14
1	68	88	77	248	652	226	249
5	147	163	189	196	555	250	171
10	161	168	121	117	284	170	67

Table 16. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in Clicks medium and supplemented with 10% mouse serum for 4 days and pulsed with Udr.

A.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	996	544	428	1468	892	865	410
1	1342	530	524	1893	1372	1132	594
5	1604	573	544	2195	1847	1352	754
10	3398	551	690	3654	2968	2252	1510

B.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W30	W867	W876	W938	W939	Mean cpm	S.E.M.
0	867	206	132	128	873	441	392
1	794	1121	213	935	857	784	342
5	1550	2223	273	1069	1840	1391	753
10	1851	2191	257	1631	1825	1551	750

Table 17. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen PHA. PBLs were cultured in Clicks medium and supplemented with 10% woodchuck serum for 4 days and pulsed with Udr.

A.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	305	44	37	48	62	99	115
1	433	49	18	56	59	123	173
5	624	49	51	47	54	165	256
10	559	21	44	47	31	140	234

B.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W30	W867	W876	W938	W939	Mean cpm	S.E.M.
0	729	680	963	470	753.	719	176
1	694	613	876	408	765	671	176
5	850	624	712	492	690	673	130
10	851	664	704	538	603	672	118

Table 18. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen PHA. PBLs were cultured in Clicks medium and supplemented with 10% mouse serum for 4 days and pulsed with Udr.

A.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	51	49	70	98	95	72	23
1	200	512	134	356	732	387	242
5	137	326	178	583	473	339	190
10	244	417	224	817	798	500	290

B.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W30	W867	W876	W938	W939	Mean cpm	S.E.M.
0	49	86	105	26	41	61	32.
1	261	386	293	124	256	264	94
5	370	410	374	521	391	413	62
10	189	173	96	302	153	182	75

Table 19. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in Clicks medium and supplemented with 0.5% woodchuck serum for 4 days and pulsed with Udr.

A.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	1568	70	875	71	207	558	655
1	1057	211	1524	155	734	736	578
5	1333	243	1972	194	918	932	751
10	1097	200	1852	193	373	743	722

B.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W30	W867	W876	W938	W939	Mean cpm	S.E.M.
0	50	20	18	93	37	43	30
1	51	34	36	163	217	100	84
5	63	37	45	190	295	126	113
10	62.	59	56	189	48	83	59

Table 20. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in Clicks medium and supplemented with 0.5% mouse serum for 4 days and pulsed with Udr.

A.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	51	49	70	98	95	72	23
1	247	328	122	712	638	409	254
5	117	106	246	837	876	436	387
10	104	109	167	806	760	389	360

B.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W30	W867	W876	W938	W939	Mean cpm	S.E.M.
0	49	86	105	26	41	61	32
1	746	812	663	403	761	677	162
5	911	1024	1231	723	902	958	186
10	366	416	211	202	315	302	94

Table 21. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen PHA. PBLs were cultured in Clicks medium and supplemented with 0.5% woodchuck serum for 4 days and pulsed with Udr.

A.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	75	70	875	71	247	268	347
1	567	257	1629	130	358	588	603
5	939	234	1557	256	636	724	549
10	825	224	1574	208	33	573	634

B.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W30	W867	W876	W938	W939	Mean cpm	S.E.M.
0	50	20	18	93	37	43	30
1	106	40	98	231	105	116	69
5	400	206	289	440	102	287	138
10	237	183	164	367	543	299	157

Table 22. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen PHA. PBLs were cultured in Clicks medium and supplemented with 0.5% mouse serum for 4 days and pulsed with Udr.

A.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	216	127	107	278	73	160	84
1	116	285	317	2539	234	908	989
5	2446.	643	394	2540	280	1261	1133
10	2104	747	466	2281	188	1157	967

B.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W30	W867	W876	W938	W939	Mean cpm	S.E.M.
0	70	132	206	44	128	116	62
1	250	213	1121	182	935	54	450
5	196	73	2223	255	1069	852	1004
10	107	57	2191	139	1631	825	1011

Table 23. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A in the presence of 2-ME. PBLs were cultured in Clicks medium and supplemented with 0.5% woodchuck serum for 4 days and pulsed with Udr.

A.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	51	49	70	98	95	72	23
1	200	512	134	356	732	387	242
5	137	326	178	583	473	339	190
10	244	417	224	817	798	500	290

B.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W30	W867	W876	W938	W939	Mean cpm	S.E.M.
0	49	86	105	26	41	61	32
1	261	386	293	124	256	264	94.
5	370	410	374	521	391	413	62
10	189	173	96	302	153	182	75

Table 24. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen Con A in the absence of 2-ME. PBLs were cultured in Clicks medium and supplemented with 0.5% woodchuck serum for 4 days and pulsed with Udr.

A.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	216	127	107	278	73.50	160	84
1	1019	413	580	2522	2098	1326	936
5	3555	834	693	2797	11648	3905	4501
10	1695	855	155	1068	2612	1277	926

B.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W30	W867	W876	W938	W939	Mean cpm	S.E.M.
0	70	132	206	44	128	116	62
1	1408	286	230	206	2369	899	964
5	1292	687	2567	310	3617	1694	1374
10	1538	465	2393	253	3356	1601	1305

Table 25. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen PHA in the presence of 2-ME. PBLs were cultured in Clicks medium and supplemented with 0.5% woodchuck serum for 4 days and pulsed with Udr.

A.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	51	49	70	98	95	72	23
1	247	328	122	712	638	409	254
5	117	106	246	837	876	436	387
10	104	109	167	806	760	389	360

B.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W30	W867	W876	W938	W939	Mean cpm	S.E.M.
0	49	86	105	26	41	61	32
1	746	812	663	403	761	677	162
5	911	1024	1231	723	902	958	186
10	366	416	211	202	315	302	94

Table 26. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen PHA in the absence of 2-ME. PBLs were cultured in Clicks medium and supplemented with 0.5% woodchuck serum for 4 days and pulsed with Udr.

PHA Concn.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	259	713	337	483	314	421	182
0.25	3809	1239	917	1367	377	1542	1323
0.5	4496	2651	1910	2056	389	2300	1483
1	7281	11700	3636	3989	1525	5626	3971
5	1459	7762	4684	954	1372	3246	2933
10	1776	4772	2617	1354	1430	2390	1422
20	1004	4433	1014	462	340	1450	1695

Table 27. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from 5 chronically infected animals in response to the mitogen PHA. PBLs were cultured in Clicks medium and supplemented with 0.5% woodchuck serum and 2-ME for 4 days and pulsed with Udr.

PHA Concn.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W859	W867	W876	W938	W939	Mean cpm	S.E.M.
0	128	144	67	121	28	98	48
0.25	601	480	353	614	96	429	214
0.5	776	891	427	603	116	562	305
1	1725	1573	684	1381	727	1218	483
5	933	999	311	666	1071	796	311
10	731	593	193	473	99	418	266
20	541	291	84	296	87	259	188

Table 28. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from 5 uninfected animals in response to the mitogen PHA. PBLs were cultured in Clicks medium supplemented with 0.5% woodchuck serum and 2-ME for 4 days and pulsed with Udr.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	259	713	337	483	314	421	182
1	4544	2260	2124	724	365	2003	1647
5	4260	4557	2705	3764	846	3226	1505
10	3695	3211	3100	3812	877	2939	1192
20	3099	4063	2417	3402	803	2757	1242

Table 29. Mean counts per minute (cpm) and standard error of the means (S.E.M.) obtained using PBLs from 5 chronically infected animals in response to the mitogen Con A. PBLs were cultured in Clicks medium supplemented with 0.5% woodchuck serum and 2-ME for 4 days and pulsed with Udr.

ConA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W859	W867	W876	W938	W939	Mean cpm	S.E.M.
0	128	144	67	121	28	98	48
1	744	624	147	803	108	485	333
5	888	1146	273	764	346	683	369
10	1620	1411	375	651	789	969	525
20	1275	1465	781	842	612	995	358

Table 30. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from 5 uninfected animals in response to the mitogen Con A. PBLs were cultured in Clicks medium supplemented with 0.5% woodchuck serum and 2-ME for 4 days and pulsed with Udr.

A.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	30	87	77	32	16	48	31
1	152	473	410	128	87	250	177
5	100	479	584	60	89	262	248
10	97	142	327	27	54	129	118

B.

PHA Conc.ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W859	W867	W876	W938	W939	Mean cpm	S.E.M.
0	31	14	22	19	41	25	10
1	2776	68	86	78	127	627	1201
5	2944	54	63	40	122	644	1286
10	2144	36	21	15	64	456	943

Table 31. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen PHA. PBLs were cultured in Clicks medium and supplemented with 0.5% woodchuck serum and 2-ME for 7 days and pulsed with Udr.

A.

PHA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	161	166	143	296	111	175	70
1	256	83	147	104	76	133	74
5	253	113	233	131	89	164	74
10	159	126	144	158	108	139	21

B.

PHA Conc.ug/ml	Tdr Incorporation (mean cpm) into PBLs (woodchuck number)						
	W859	W867	W876	W938	W939	Mean cpm	S.E.M.
0	212	183	106	273	149	184	63
1	145	87	164	268	34	139	88
5	179	85	109	235	30	127	80
10	223	98	85	271	69	149	91

Table 32. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen PHA. PBLs were cultured in Clicks medium and supplemented with 0.5% woodchuck serum and 2-ME for 4 days and pulsed with Tdr.

A.

PHA Conc.ug/ml	BrdU Incorporation (mean cpm) into PBLs (woodchuck number)						
	W26	W935	W936	W937	W933	Mean cpm	S.E.M.
0	53	58	20	220	164	103	84
1	164	186	72	497	646	313	245
5	402	310	80	472	993	451	336
10	291	226	68	1231	989	561	514

B.

PHA Conc.ug/ml	BrdU Incorporation (mean cpm) into PBLs (woodchuck number)						
	W859	W867	W876	W938	W939	Mean cpm	S.E.M.
0	158	132	76	68	65	100	42
1	298	551	99	85	220	250	189
5	636	747	153	410	200	429	261
10	302	262	71	44	157	167	113

Table 33. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from A) 5 chronically infected animals and B) 5 uninfected animals in response to the mitogen PHA. PBLs were cultured in Clicks medium and supplemented with 0.5% woodchuck serum and 2-ME for 4 days and pulsed with BrdU.

PHA concn. ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)											
	W405	W360	W363	W414	W507	W518	W526	W527	W540	W556	Mean cpm	S.E.M.
0	40	23	38	18	23	39	34	13	32	50	31	11
1	75	80	163	127	229	469	266	134	320	176	204	121
5	400	521	771	623	697	644	575	137	361	601	521	181
10	333	322	621	188	78	233	80	26	252	582	271	202

Table 34. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from 10 chronically infected animals in response to the mitogen PHA. PBLs were cultured in Clicks medium and supplemented with 0.5% woodchuck serum and 2-ME for 4 days and pulsed with Udr.

PHA concn. ug/ml	Udr Incorporation (mean cpm) into PBLs (woodchuck number)											
	W939	W406	W407	W524	W867	W32	W17	W520	W859	W30	Mean cpm	S.E.M.
0	28	32	67	20	30	65	15	20	32	24	33	18
1	727	380	373	203	503	990	116	256	596	89	423	286
5	1071	963	842	282	591	1008	225	1103	634	268	699	347
10	99	181	129	62	223	314	56	171	382	207	182	105

Table 35. Mean counts per minute (cpm) and standard error of the mean (S.E.M.) obtained using PBLs from 10 uninfected animals in response to the mitogen PHA. PBLs were cultured in Clicks medium and supplemented with 0.5% woodchuck serum and 2-ME for 4 days and pulsed with Udr.