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HLA EXPRESSION IN HEPATOCELLULAR CARCINOMA CELL LINES

BY

KEREN ANNE COPLAN

Being a dissertation presented in fulfilment of the requirements governing the degree of Masters of Science in the Faculty of Medicine, University of the Witwatersrand.

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Abstract

Recent investigations have shown enhanced or aberrant expression of major histocompatibility system (MHC) antigens on cells lines derived from human hepatocellular carcinoma (HCC) **in vitro** and HCC **in vivo**. The present study undertook to further clarify the biological significance of MHC antigen expression in HCC and to elucidate the role of potential modulating agents of antigen expression. These studies have utilised several hepatic tumour-derived cell lines as **in vitro** model systems. The cell lines included PLC/PRF/5 (Alexander cell line), Hep 3B, Hep G2, TONG PHC, HA22T/VGH, HA59T/VGH and Mahlavu. Two cell lines, K562 and Raji, were used as negative and positive controls respectively. The cell line K562, a B-lymphoid derived cell line, expresses negligible amounts of MHC complex while Raji, an erythromyeloid derived cell line, expresses both MHC Class I and II antigens as well as their respective invariant chains, ß2-microglobulin and Ii. The expression of surface MHC was quantitatively assessed by the indirect immunofluorescence indirect immunofluorescence (IIF) assay. The results of this assay, indicated that all hepatic tumour-derived cell lines exhibit spontaneous MHC Class I surface antigens while only two poorly differentiated cell lines, HA22T/VGH and HA59T/VGH, display MHC Class II antigens. The validity of the assignment of Raji and K562 as positive and negative

control cell lines was confirmed by this assay. The quantitative assessment of MHC surface antigen expression was achieved by the enzyme-linked immunosorbent assay (ELISA). Dose response experiments performed on HCC cell lines confirmed spontaneous surface expression of MHC Class I antigens and MHC Class II antigen expression on the HA22T/VGH and HA59T/VGH cell lines. In addition, the Alexander cell line displayed MHC Class II surface antigens using the ELISA but not the IIF technique. The effects of modulating agents such the interferons alpha and gamma, sodium butyrate and clofazimine on MHC expression were then investigated on the cell lines HA22T/VGH, HA59T/VGH and TONG PHC. The results indicate that gamma interferon and clofazimine are modulators of MHC Class II expression while sodium butyrate used at a physiological concentration enhances MHC surface antigen expression. cDNA probes directed against MHC Class I, Class II and its associated invariant chain were isolated and purified.

This is to certify that the dissertation "HLA EXPRESSION IN HEPATOCELLULAR CARCINOMA CELL LINES" presented for the degree of Masters of Science at the University of the Witwatersrand, Johannesburg, is my own unaided work and has not been presented at any other university.

...K Coplan..... ...15.day of. August..,1991

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B.Sc. (HONS)

Permission to work with human subjects was obtained from the Human Ethics Committee, University of the Witwatersrand, Johannesburg. (Clearance Certificate No. 29/5/89 (Document A79/55).

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS
(REF. R14/49)

CLEARANCE CERTIFICATE PROTOCOL NO: 7/5/89

PROJECT : MOLECULAR INVESTIGATIONS ON THE EXPRESSION OF HLA MOLECULES ON HEPATOMA CELL LINES. (HLA EXPRESSION IN HEPATOCELLULAR CARCINOMA CELL LINES).

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DATE CONSIDERED 26/5/89

RECOMMENDATION OF COMMITTEE :

APPROVED [X]

Date : 29/5/89 CHAIRMAN : .....
Professor P E Cleaton-Jones

Guidelines for written "Informed Consent" attached where applicable.

DECLARATION BY INVESTIGATOR/S
To be completed in duplicate and ONE copy returned to Miss S M Boshoff, Office of the Deputy Registrar (Research), Room 10002, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/~~we~~ are authorised to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions.

Should any departure be contemplated from the research procedure as approved I/we undertake to resubmit the Protocol to the Committee.

DATE : 12.6.89 SIGNED : .....

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1. Graduated with the degree of B.Sc., University of Cape Town, December 1986.

2. Graduated with the degree of B.Sc. Hons. Chemistry, University of Cape Town, December 1987.

3. The research on which this dissertation is based was commenced in April 1989 and completed in December 1990.

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Abbreviations

AFP	Alpha fetoprotein
APC	Antigen presenting cell
BP	base pair
ß2m	Beta-2-microglobulin
BSA	Bovine serum albumin
CsCl	Cesium chloride
CLD	Chronic liver disease
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribonucleic Acid
cDNA	Complementary DNA
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified eagle's medium
ER	Endoplasmic reticulum
ELISA	Enzyme-linked Immunosorbent Assay
EIA	Enzyme immunoassay
FCS	Fetal calf serum
eg	for example
HBV	Hepatitis B Virus
HBsAg	Hepatitis B surface antigen
HCC	Hepatocellular Carcinoma
H-2	Histocompatibility 2
HLA	Human Leukocyte Antigen
IIF	Indirect immunofluorescence

Ii	Invariant chain
MHC	Major Histocompatibility Complex
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
MOAB	Monoclonal Antibody
NK	Natural killer
OD	Optical density
PAP	Peroxidase anti-peroxidase
PBS	Phosphate buffered saline
2-d	Two-dimensional
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
mRNA	Messenger RNA
rRNA	Ribosomal RNA
RER	Rough endoplasmic reticulum
TCR	T cell receptor
ie	that is

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CHAPTER 1.

INTRODUCTION

1.1. HISTORY OF THE MHC.

The elucidation of the structure and mechanisms of the products of the Major Histocompatibility Complex (MHC) and the understanding of their crucial role in regulating the immune response, has been an area of intensive research over the past two decades (Gopas et al 1989). In 1938, Gorer discovered the first evidence of a histocompatibility system in the mouse (Klein 1986). The histocompatibility molecules could be recognised for two reasons. Firstly, it was possible to produce antisera to the molecules and secondly, the molecules were capable of inducing rejection in the (cells of) tissues of incompatible mice (Klein 1979). The above property governs tissue compatibility which is important in tissue transplantation (Klein 1986). Gorer, Lyman and Snell designated the molecules as histocompatibility antigen and gave them the serial number two (Klein 1979). The term histocompatibility was coined by Snell in 1948 (Marx 1980) to denote the cell surface antigens that determine whether one tissue is compatible with another. Gorer and Snell designated the gene coding for the antigens as H-2 (Histocompatability-2) (Marx 1980). Dausset was the first to demonstrate the existence

of human leukocytes that carried group specific characteristics that were called isoantigens (allo-antigens). Dausset designated the leucocyte group as Mac (Dausset 1958).

These antigens appeared to be genetically controlled as was shown in studies on monozygotic twins (Dausset & Brecy 1957, Lalezari & Spaet 1959), families and in the appearance of maternal alloantigens during pregnancy (Payne & Rolfs 1958). In 1958, Payne and Rolfs demonstrated the existence of leukocyte factors in pregnant women who had not received prior blood transfusions by leukoagglutinin formation in the mother. At the same time, van Rood demonstrated leukocyte antibodies in sera from pregnant women and provided data to show the existence of a leukocyte isoantigen locus which he designated as four (van Rood et al 1958). This locus was found on the basis of a procedure for testing leukocyte agglutination. Van Rood found that the locus was occupied by one of two alleles, 4^a or 4^b (van Rood & van Leeuwen 1963). Family studies proved that 4^a and 4^b were inherited as simple Mendelian autosomal co-dominant alleles (van Rood & van Leeuwen 1963). The alleles 4^a and 4^b are now known as Bw4 and Bw6. Similarly, Payne defined a different allelic system called LA (Leukocyte Antigen) (Zmijewski et al 1970). The two systems LA and Four were shown to be

closely linked (Zmijewski et al 1970).

During this time, in the 1960's, the situation concerning leukocyte antigens was highly confused. Many more antigen systems were identified and it was not clear how they were related or indeed whether they actually were related in any way (Marx 1980). It was only in 1964 during the first International HLA Workshop that the development of the HLA (Human Leukocyte Antigen) system was clarified (Bodmer 1977). Subsequent workshops, organised biennially, involved an exchange of sera, reagents and data from an increasing number of laboratories. The workshops 1-6 starting 1964 to 1975 defined the HLA-A,B,C loci as a universal phenomenon (Bodmer 1977).

In 1968, the World Health Organisation (WHO) assigned the name HL-A to the leukocyte Group 4 system (Zmijewski et al 1970). It was thought that the HL-A haplotype consisted of one genetic determinant (or blank) from the LA series and one from the four series (Bodmer 1972). In 1975, during the sixth workshop, the HLA-D locus was accepted as a system identified by mixed lymphocyte culture techniques. Following that, in 1975, the WHO announced that "HLA" was to define the whole region while HLA-A, HLA-B, HLA-C and HLA-D were to refer to the individual loci within the entire region (Bodmer 1977).

The HLA-D region, encoding class II antigens, was first found using studies of Mixed Leukocyte Culture (MLC) (Bach & Hirschorn 1964, Bain et al 1964). In the MLC or Mixed Lymphocyte Reaction (MLR), lymphocytes of two individua' are incubated together and they respond by undergoing clonal proliferation and lymphokine production. The amount of proliferation is a measure of incompatibility or alloreactivity between the lymphocyte populations of two individuals (Korman et al 1985).

Amos and Bach suggested in 1968, that a genetic region existed that encoded molecules stimulating proliferation in MLC, that may be linked to but were different from the HLA-A and -B loci, that is a region such as HLA-D. (Amos & Bach 1968). They presented data that the HLA-D related products could be recognised by serology using an MLC (Amos & Bach 1968).

HLA is a codominant system as both alleles are expressed equally on the cell surface. Each antigen is identified by a letter for the locus which controls it, followed by a number defining the particular specificity of that locus.

The letter w following a locus symbol and preceding the number, indicates that a specificity is only provisionally identified and is undergoing international "workshop"

confirmation in which several laboratories participate to identify a particular antigen (Barrett 1988). There are 24 known alleles at the A locus, 52 at the B locus and 11 at the C locus. There are 20 alleles at the HLA-DR and 9 at the DQ locus. All the above loci are identified serologically by the cellular typing technique known as the microcytotoxicity test (Barrett 1988). Twenty six alleles have been recognised at the Dw locus and 6 at the DP locus. (WHO Nomenclature Committee 1988)

Table 1.1 lists the HLA-A,B,C and D locus antigens identified to date (WHO Nomenclature Committee 1988). The listing of the specificities tabled in Table 1.1 will be updated as more undefined alleles at the HLA-A loci and all the alleles at the B,C, Dw, DR loci as well as the DP and DQ loci are identified in the coming years.

1.2. THE STRUCTURE OF THE MHC

The loci controlling the two major sets of serologically defined antigens, the HLA-A,B,C (Class I) determinants and the HLA-D region (Class II) determinants, have been shown to be closely linked to each other on the short arm of chromosome 6 (Bodmer 1987) (Figure 1.1). Situated on chromosome 6 as well, the genes encoding for several complement factors, C4A, C4B, C2, and factor B (Bf) known as MHC Class III have also been identified (Barrett 1988).

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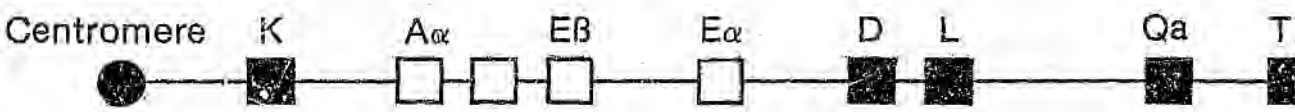
The HLA and its murine analogue, the H-2 system, display marked serological, functional and structural homologies (Klein 1979). The H-2 complex, located on chromosome 17 (Klein 1979) is divided into six regions (K, I, S, G, D and T) (Figure 1.1). The I region is divided into five subregions (A, B, J, E and C). The murine equivalent to HLA-A,B,C is H-2K, H-2L and H-2D (MHC Class I). The regions H-2 IA and IE/C encode biochemically identified Ia polypeptides which are equivalent to the HLA-D region antigens (Klein 1979). A major feature of these genetic regions is the high degree of polymorphism expressed (Klein 1979, Bodmer & Bodmer 1978). The HLA region as a whole, though it may comprise only one-thousandth of the human genome, includes a thousand or more genes with a large series of multiple alleles at each of several different although unrelated loci (Bodmer & Bodmer 1978).

The H-2K and 2D gene products and the HLA-A, B, C antigens have the same structure and are associated with ß2-microglobulin (ß2m) (see Fig. 1.2) (Klein 1979). The class I antigens (human HLA-A, B,C and the murine H-2K, -2D and -2L) are known as the classic transplantation antigens (Kaufman & Strominger 1983). Also, homologous molecules with identical tissue distribution (primarily restricted to B lymphocytes and macrophages) and having similar functions

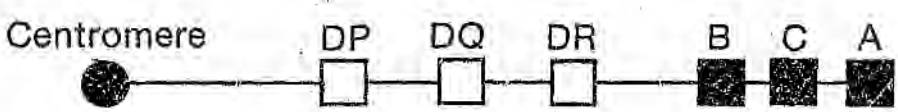
TABLE 1.1: COMPLETE LISTING OF RECOGNISED HLA SPECIFICITIES[a]

A	B	C	D	DR	DQ	DP
A1	B5	Cw1	Dw1	DR1	DQw1	DPw1
A2	B7	Cw2	Dw2	DR2	DQw2	DPw2
A3	B8	Cw3	Dw3	DR3	DQw3	DPw3
A9	B12	Cw4	Dw4	DR4	DQw4	DPw4
A10	B13	Cw5	Dw5	DR5	DQw5(w1)	DPw5
A11	B14	Cw6	Dw6	DRw6	DQw6(w1)	DPw6
Aw19	B15	Cw7	Dw7	DR7	DQw7(w3)	
A23(9)	B16	Cw8	Dw8	DRw8	DQw8(w3)	
A24	B17	Cw9(w3)	Dw9	DR9	DQw9(w3)	
A25(10)	B18	Cw10(w3)	Dw10	DRw10		
A26(10)	B21	Cw11	Dw11(w7)	DRw11(5)		
A28	Bw22		Dw12	DRw12(5)		
A29(w19)	B27		Dw13	DRw13(w6)		
A30(w19)	B35		Dw14	DRw14(w6)		
A31(w19)	B37		Dw15	DRw15(2)		
A32(w19)	B38(16)		Dw16	DRw16(2)		
Aw33(w19)	B39(16)		Dw17(w7)	DRw17(3)		
A34(10)	B40		Dw18(w6)	DRw18(3)		
Aw36	Bw41		Dw19(w6)			
Aw43	BW42		Dw20	DRw52		
Aw66(10)	B44(12)		Dw21			
Aw68(28)	B45(12)		Dw22	DRw53		
Aw69(28)	Bw46		Dw23			
Aw74(w19)	Bw47		Dw24			
	Bw48		Dw25			
	B49(21)		Dw26			
	Bw50(21)					
	B51(5)					
	Bw52(5)					
	Bw53					
	Bw54(w22)					
	Bw55(w22)					
	Bw56(w22)					
	Bw57(17)					
	Bw58(17)					
	Bw59					
	Bw60(40)					
	Bw61(40)					
	Bw62(15)					
	Bw63(15)					
	Bw64(14)					
	Bw65(14)					
	Bw67					
	Bw71(w70)					
	Bw70					
	Bw72(w70)					
	Bw73					
	Bw75(15)					
	Bw76(15)					
	Bw77(15)					
	Bw4					
	Bw6					

[a] WHO Nomenclature Committee 1988



Structural genes for the murine major histocompatibility are situated on chromosome 17. The class I H2-K histocompatibility genes are separated from the H2-D and H2-L genes.



The structural genes for the human major histocompatibility complex are on chromosome 6. The class I genes HLA-A, -B and -C are grouped, unlike the murine MHC.

Class I Class II

Figure 1.1 : Redrawn from Barrett (1988)

were found in antigens encoded by the murine I regions, the Ia antigens, and in man, by the HLA-D region (Humphreys et al 1976).

Knowledge in the field of HLA molecular genetics has progressed rapidly over the past few years (Jordan et al 1985). It has been shown using sequencing data, that the HLA-A,B,C, D region products are members of the immunoglobulin super-gene family (Bodmer 1987). Thus, the HLA (human and H-2 (murine) major histocompatibility products have been called histoglobulins (Klein 1979).

1.2.1 MHC CLASS I

The HLA-A, B, C loci lie at the end of the HLA region on chromosome 6 (See Fig 1.1). The typical HLA-A, B, C gene produces a molecule with three extracellular domains: alpha1, alpha2 and alpha3 of a molecular weight of 45 kDa (Vega & Strominger 1989). Of these three domains alpha3 is the most conserved, whereas alpha1 and alpha2 bear polymorphic N-terminal determinants (Vega & Strominger 1989). This was observed from the elucidation of the crystal structure of HLA-A2 (Bjorkman et al 1987a, 1987b). The molecule also consists of a transmembrane segment and a short cytoplasmic conserved immunoglobulinlike C-terminal region or "tail" (See Fig 1.2) (Kaufman & Strominger 1982). All these products are non-convalently associated

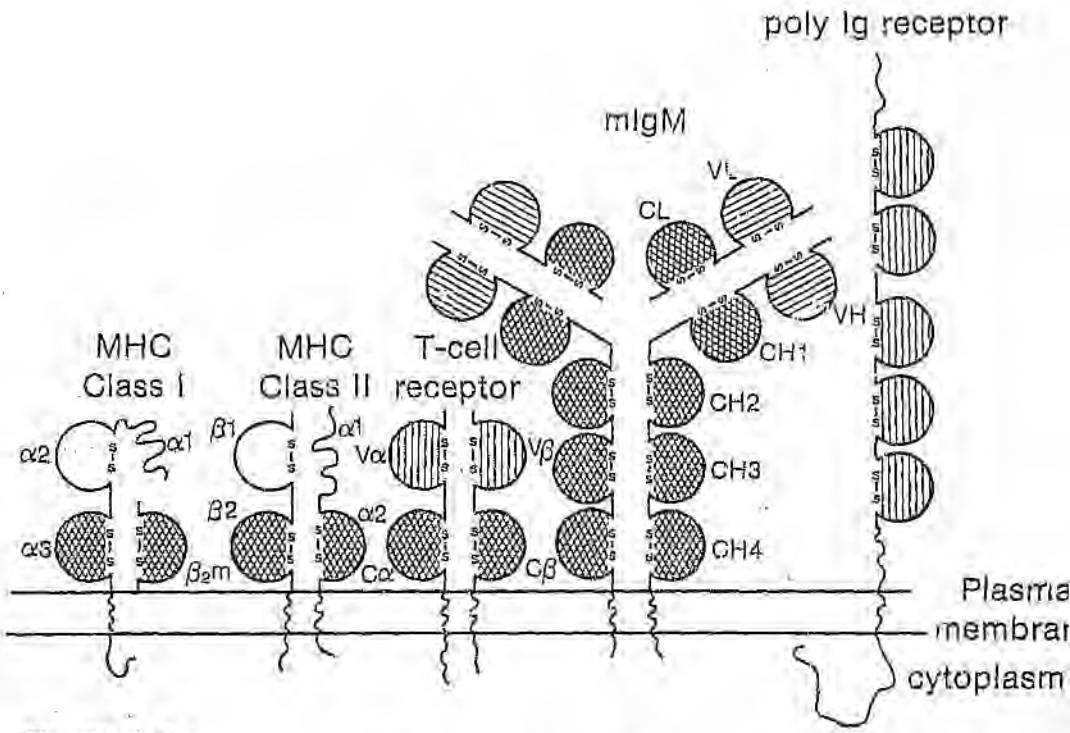


Figure 1.2 :
Schematic representation of MHC antigens and other membrane proteins with homology to immunoglobulin constant domains (▒▒▒) and variable domains (|||||||).
(redrawn from Auffray and Strominger (1986))

with ß2-microglobulin (ß2m) which is encoded for by a single gene on chromosome 15 (Goodfellow et al 1975) The ß2-microglobulin, an ivariant or highly conserved 12kDa polypeptide, lies outside the cell membrane and associates particularly with the alpha3 domain as shown in Figure 1.2. (Bjorkman et al 1987a). The conserved membrane-proximal alpha3 and ß2m domains also exhibit a similar tertiary structure that has been described for the antibody domains)the constant region) of the immunoglobulins (Bjorkman et al 1987a). The alpha3 and ß2m domains are paired by a novel interaction not found between pairs of constant domains in the known antibody structures (eg CH1 and CL in an Fab fragment) (Bjorkman et al 1987a). there is a significant amino acid sequence homology between alpha3, ß2m and the constant regions of the immunoglobulins (Orr et al 1979, Tragardh et al 1979). The alpha1, alpha3 and ß2m domains are stabilised by an intrachain disulphide bonds. (See Fig 1.2).

1.2.2. MHC CLASS II

The MHC Class II or HLA-D region is defined by its complexity at both the genetic and serological levels (Trowsdale et al 1985, Cresswell et al 1987). The extensive review of Trowsdale et al (1985) of genetic studies on the characterisation and polymorphism of the HLA-D region alpha and ß chain gene, suggests that there are at least three major sets of products: HLA-DR, -DP (formerly SB) and -DQ (previously DC) all having a very similar structure (Trowsdale et al 1985).

Each antigen consists of a heterodimer, having its own alpha and ß subunit. The HLA-D locus has revealed at least 6 alpha chain genes and at least 7 ß chain genes, nearly all assigned to DR, DQ or DP (Trowsdale et al 1985). The alpha and ß chains are transmembrane glycoproteins of approximately 35kDa and 27kDa respectively (Trowsdale et al 1985). The subunits are highly polymorphic and are non-convalently associated (Steinhoff 1990). The alpha and ß subunits have a short cytoplasmic immunoglobulin-like conserved C-terminal tail (Kaufman & Strominger 1982). The polypeptide subunits span the lipid bilayer and protrude into the extracytoplasm of the cell (Walsh & Crumpton 1977, Kaufman & Strominger 1979). The N-terminus of the extracytoplasmic portions of both chains is recognisably divided into two domains (Trowsdale et al 1985). Both domains of the ß subunit (See Fig 1.2) contain an intrachain sulphydryl bond (Cresswell et al 1987). Only the membrane-proximal alpha2 domain of the alpha subunit has an internal disulphide bond (Cresswell et al 1987). The membrane-proximal domains of both subunits alpha2 and ß2 exhibit a limited amino acid sequence homology with the immunoglobulin family and are highly conserved (Kaufman & Strominger 1983).

1.2.2.1 THE ASSOCIATION OF MHC CLASS II WITH A THIRD GLYCOPROTEIN.

1.2.2.1.1. Discovery of the HLA-D Region-Associated Invariant Chain.

In 1979, Jones et al by using 2-dimensional (2-d) gel electrophoresis of I-A and I-E subregion antigens of the H-2 complex demonstrated that all Ia antigen immunoprecipitates contain three distinct polypeptide chains (Jones et al 1979). The third polypeptide was identified to be an invariant Ia-associated chain because it lacked polymorphism at an isoelectric point as identified by 2-d gel electrophoresis (Jones et al 1979). The Ia-associated invariant chain was termed Ii indicating Murine MHC Class II and 'i' for 'invariant'. Similar findings were reported by Charron and McDevitt (1979) who also identified the invariant chain to be of approximately 32 kDa (Charron & McDevitt 1980).

Since 1980 many abbreviations have been used to describe the HLA-D region-associated invariant chain, including Ii (Jones et al 1979), p31 (Owen et al 1981), Gamma (Kvist et al 1982, Claesson & Peterson 1983) and In (Long 1985). For the purpose of this study, the name Ii will be used.

1.2.2.1.2 STRUCTURE OF Ii

Since the discovery of Ii, the functional role of the HLA-D

region associated-invariant chain has been a subject of intense investigation with few answers having been provided (Long 1985, Hämmerling & Moreno 1990). Structurally, the invariant chain is a basic glycoprotein of approximately 31kDa or 41kDa which originates from differential RNA splicing (Yamamoto et al 1985a). It is encoded by a gene not linked to the MHC genes but located on chromosome 18 in mice and chromosome 5 in humans (Yamamoto et al 1985b, Glasson-Welsh et al 1984). The invariant chain is an unusual transmembrane glycoprotein since it is inverted compared to most membrane proteins (such as MHC Class II) with the C-terminus in the extra-cytoplasm (Cresswell et al 1987). No homology with any known protein, including the immunoglobulin, MHC Class II, MHC Class I or the MHC Class I-associated invariant chain, β2-microglobulin, is evident in the primary Ii amino acid sequence (Cresswell et al 1987).

Its uncommon configuration of a N-terminal cytoplasmic portion and an extracytoplasmic C-terminal 'tail' is shared by a limited number of integral glycoproteins (Fields et al 1981, Drickamer & Mamon 1982) such as the transferrin receptor (McClelland et al 1984). Cresswell (1985) suggests that the endocytic pathway followed by the latter intersects the exocytic route taken by newly synthesised MHC Class II antigens prior to the dissociation of the Ii

chain before the Class II molecule is expressed on the plasma membrane.(See Section 1.3.2.1.3.2.1) This intracellular interaction between Class II molecules and foreign proteins may be important in Class II antigen function (Cresswell 1985).

1.3 THE FUNCTION OF THE MHC.

1.3.1 History of the Function of the MHC

In 1962, Gowans et al postulated that the immune response may be initiated by the interaction of antigens and lymphocytes. Miller (1962) and Good et al (1962) discovered that lymphocytes differentiate into two classes of cells which are either derived from the thymus (T lymphocytes) or Bursa of Fabricus (B lymphocytes). Warner et al (1962) identified two distinct immunocompetent lymphoid-derived populations and recognised that cells derived from the thymus were responsible for cellular immune phenomena. It was also demonstrated that immunogenicity is regulated by helper T-cells and cytotoxic T-cells (Rajewsky et al 1969, Kapp et al 1974). Katz and Benacerraf (1972) postulated that helper T-cells' responses were regulated by Ir (immune-response) genes in mice. At this stage, it had become increasingly obvious that the immune system was complex and that T lymphocytes had a critical role to play in the regulation of immunity (Benacerraf 1981).

The work of Benacerraf, Katz and others on cellular interactions between histoincompatible T and B lymphocytes showed that a dual specificity of thymus-derived lymphocytes for structures coded by the MHC (self) and for foreign antigens (non-self) existed in mice (Katz et al 1970, Katz et al 1971, Katz et al 1973a). In these studies histoincompatible lymphocytes were used in order to avoid an allogeneic effect (Katz et al 1973a). The word histoincompatible implies that lymphocytes were derived from different murine stains. Furthermore it was shown that the co-operative interaction of T and B lymphocytes is restricted by the H-2 gene complex (Kindred & Schreffler 1972, Katz et al 1973b). Genes within the I-region of the MHC appear to control the activity of helper T-cells (Green et al 1966, Katz et al 1973c) whilst Ia molecules determine immune responsiveness to T-cell antigens (Kappler & Marrack 1978).

1.3.2 DEMONSTRATION OF THE FUNCTION OF THE MHC

Originally, Unanue & Askonas (1968) demonstrated that macrophage-bound antigen exhibited enhanced immunogenicity which persisted long after the uptake of antigen by macrophage. Unanue and Cerottini (1970) found that when keyhole limpet hemocyanin was used as an antigen carrier it was taken up by the macrophage, rapidly catabolised and eliminated from the cell. However, they demonstrated that

the antigen still remained immunogenic by virtue of a few molecules of residual antigen bound to the plasma membrane of the macrophage (Unanue & Cerottini 1970). Later it was demonstrated that the involvement of the membrane glycoproteins encoded in the major histocompatibility gene complex are essential for the development of an immune response in most cellular interactions (Babbitt et al 1985, Rosenthal & Shevach 1973a, 1973b).

1.3.2.1 FUNCTION OF MHC CLASS II

1.3.2.1.1 Demonstration of Antigen Processing and Presentation.

Rosenthal and Shevach (1973a, 1973b), using a guinea pig as a model, demonstrated that macrophages contribute to the specificity of a T-cells response after its activation by macrophage-bound antigens. They also demonstrated that the Ia molecules, are essential for accessory cells to present antigenic peptides to helper T-lymphocytes (Rosenthal & Shevach 1973a, 1973b). These elegant studies indicated a role for the macrophage in events controlled by the I region of the MHC and demonstrated an essential interaction between macrophage and T-helper lymphocytes (Unanue 1981). Further studies by Ziegler and Unanue (1981) using the bacterium **Listerium monocytogenes** showed that an intracellular mechanism or processing (Unanue & Cerottini 1970) of the antigen **Listerium monocytogenes** by

macrophages was required for the expression or presentation of antigen on macrophage and subsequent recognition by T cells in the context of H-2^k. Studies using lysosomotropic amines, ammonia and chloroquine demonstrated the inhibition of the development of effective antigen presentation when the lysosomotropic reagents were given immediately after antigen binding to accessory cells, but not at later times (Ziegler & Unanue 1982). Chloroquine and ammonia cause the collapse of the intracellular pH gradient by raising the lysosomal pH (Ziegler & Unanue 1982). This collapse causes a decrease in proteolytic cleavage in the acidic lysosome and thereby inhibition of effective antigen presentation (Ziegler & Unanue 1982). Studies also showed that T cells recognise chemically denatured forms of antigen (Thomas DW et al 1981, Sette et al 1989[a]) as well as native globular proteins (Matis et al 1982) although the latter has since been disputed by other workers (Sette et al 1987). In 1959, Gell and Benacerraf illustrated by means of delayed hypersensitivity experiments in guinea pigs that T cells, like B cells, cannot differentiate between native and denatured antigens. Later studies indicated that small antigenic peptides can be as effective as intact protein in inducing T cell responses (Matis et al 1982, Thomas DW et al, 1981, Thomas JW et al 1981). It was also reported using the time kinetics of purified protein derivative (PPD) (Waldron

et al 1974) and Listerium monocytogenes (Ziegler & Unanue 1981) as antigen that a time lapse of 60min followed the initial binding of antigen to macrophage and effective presentation of the antigen by the accessory cell (macrophage) to T cells. All the above accumulated findings led to the postulation of antigen processing (Shimonkevitz et al 1983).

1.3.2.1.2 MECHANISMS OF ANTIGEN PROCESSING AND PRESENTATION.

The first step in antigen processing involves the internalisation or endocytosis of native globular antigen by accessory cells (such as macrophages) (Koch et al 1989) as seen in Fig 1.3. Two mechanisms of antigen uptake by accessory cells have been suggested. The first mechanism involves the pinocytosis of soluble antigen and has been demonstrated to be size dependent. The second mechanism, which appears to be more common, involves the binding of the antigen to the cell surface of the accessory cell (Chesnut & Grey 1985). This binding, which takes place prior to the endocytic event, could be via specific receptors such as lectin-like carbohydrates such as mannose or via non-specific, non-covalent interactions with undefined structures on the cell surface of the accessory cells (Chesnut & Grey 1985). After internalisation, two distinct pathways are used to process antigens depending on

which T cell response needs to be elicited (Yewdell & Bennink 1990). Cytotoxic T lymphocytes (CTLs) generally require antigen presenting cells that are biosynthesising antigen in the cytoplasm (Yewdell & Bennink 1990). In contrast, T-helper cells require a different antigen processing mechanism involving the endosomal pathway (Yewdell & Bennink 1990) which will be discussed in this section.

The initial cytoplasmic compartment involved in the pathway of soluble antigens by accessory cells is a vesicle termed an endosome (Koch et al 1989). However, it is presently unknown in which exact intracellular compartment the internalisation of antigen occurs (Koch et al 1989). Because of the proteolytic cleavage of the native proteins, acidic endosomal compartments such as lysosomes have been suggested to internalise the soluble antigen and partially degrade it to amino acids (Koch et al 1989). Cathepsin-D, a membrane-bound enzyme found in endosomes is suggested to perform the degradation (Diment et al 1988). The extent to which proteolysis is required for the presentation of antigen in context of MHC Class II molecules still needs to be established (Yewdell & Bennink 1990). It is known, however, that MHC Class II molecules present peptides mainly derived from extracellular antigens (ie exogenous antigens) to helper T lymphocytes (Babbitt et al 1985, Yewdell & Bennink 1990). The precise mechanism of how the

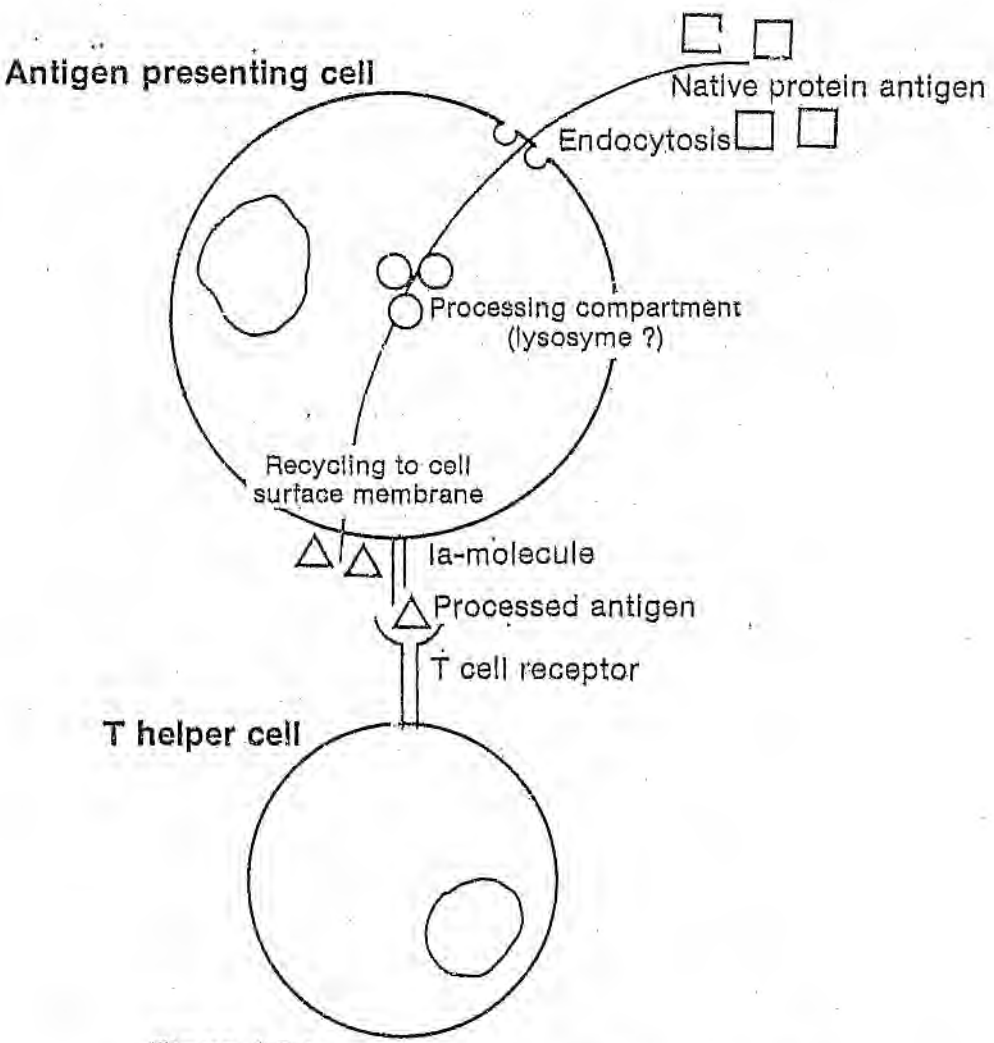


Figure 1.3 :
Model of antigen processing and presentation
(Redrawn from Buus (1987))

denatured or cleaved antigens are re-expressed in conjunction with MHC Class II antigens on the cells surface of the accessory cell is not presently understood (Owen et al. 1981, Yewdell & Bennink 1990).

Antigen processing and presentation has been shown to be an energy-requiring, dynamic process that requires cell viability (Shimonkewitz et al 1983, Unanue 1981). However, it was shown by [illegible] that proteolytic cleavage or even denaturation of the intracellular processing event taking place in the accessory cell (Shimonkewitz et al 1983,. [illegible]ng, resulting in antigen presentation by accessory cells, is necessary for T lymphocyte recognition of the antigen and the T cell's consequent activation (Koch et al 1989, Babbitt et al 1985). Babbitt et al (1986) demonstrated, in a murine model, using a defined synthetic immunogenic peptide, hen egg lysosome (HEL) as an antigen, that presentation of the antigen and the T cell's subsequent activation was restricted by Ia molecules.

1.3.2.1.3 MHC CLASS II BIOSYNTHESIS, ASSEMBLY AND PROCESSING

1.3.2.1.3.1 Biosynthesis of MHC Class II Glycoproteins. Like most proteins and glycoproteins destined for the plasma membrane, the HLA-DR region antigens are synthesised

on membrane-associated ribosomes on the rough endoplasmic reticulum (RER) (Cresswell et al 1987, Owen et al 1981). In vitro translation studies have shown that the alpha and beta chains are synthesised as precursors with a signal sequence, an N-terminal peptide of 20-30 amino acids in length, in common with many other membrane and secreted glycoproteins (Owen et al 1981). The nascent protein subsequently binds to the RER membrane via an RER protein known as a 'docking protein' (Meyer & Cresswell, 1982). As translation continues, both the signal sequence and the remainder of the protein are co-translationally assymetrically inserted through the membrane by an unknown mechanism (Owen et al 1981, Cresswell et al 1987). In the presence of a mixed microsomal system, the signal peptide is cleaved and the 'core' oligosaccharide units are added to the nascent alpha and beta subunits (Ploegh et al 1979). This above scheme is followed by Class I heavy chain (alpha3) and Class II alpha and beta subunits (Owen et al 1981). The glycoproteins are ultimately orientated with their N-terminus in the extracytoplasmic space (Owen et al 1981).

Pulse/chase labelling and 2-d gel electrophoresis have proved to be powerful techniques for the analysis of the biosynthesis and glycosylation of the HLA-DR antigens (Machamer & Cresswell 1982). In these experiments Class II

antigen-positive cells are briefly labelled (5-10 mins) with a radioactive amino acid such as 37S-methionine and then excess unlabelled or "cold" amino acid is added. Class II molecules are isolated at different time intervals during a subsequent incubation at 37°C and analysed thereafter by 2-d gel electrophoresis (Machamer & Cresswell 1982).

1.3.2.1.3.2 ASSEMBLY OF MHC CLASS II GLYCOPROTEINS.

1.3.2.1.3.2.1. Function of the Associated Invariant Chain, Ii.

Soon after the completion of the synthesis of the alpha and beta chains, the molecules complex with a third polypeptide, the gamma or Ii or invariant chain (Machamer & Cresswell 1982, Simonis et al 1989, Stockinger et al 1989). The invariant chain remains associated with the alpha-beta dimer throughout intracellular transport (Long 1985). The oligomeric Class II-Ii complex (Koch et al 1989) moves from the endoplasmic reticulum (ER) to the Golgi body where the individual chains undergo post-translational modifications (Simonis et al 1989). However, the Ii chain is released from the alpha-beta dimer after the three chains have been through the Golgi complex (Sekaly et al 1986, Miller & Germain 1986).

Although no evidence for the function of the invariant

chain exists at present (Machamer & Cresswell 1982), several possibilities including the assembly, association, and cytoplasmic transport of the alpha-beta complex to the cell surface have been suggested (Koch & Hämmerling 1982, Charron & McDevitt 1979). The latter possibility (of cytoplasmic transport) was discounted when it was shown that Ia molecules can be expressed at the cell surface after transfection of alpha and beta chain genes into cell lines that do not express endogenous Ii (Sekaly *et al* 1986, Miller & Germain 1986). Moreover, the possibility exists that Ii functions to control post-translational processing of Class II molecules without the overall level of Ia expression being affected (Miller & Germain 1986, Simonis *et al* 1989). Miller and Germain (1986) hypothesized that the function of the invariant chain is to determine the association of the antigen with the Class II molecule in the low pH environment of the endosomes or lysosomes. Their hypothesis is supported by Babbitt *et al* (1985) and Cresswell's (1985) findings that antigenic peptides physically bind to Ia molecules in certain configurations. These workers suggest that the endocytic pathway of transferrin-neuraminidase conjugates intercept with the exocytic pathway of vesicles carrying Ia-Ii complexes to the cell surface respectively.

1.3.2.1.3.3. PROCESSING OF MHC CLASS II GLYCOPROTEINS.

The association of Ii with Ia molecules has been shown to be transient (Long 1985). As mentioned earlier, the three chains (alpha, beta, Ii) are assembled in the endoplasmic reticulum (ER) (Owen et al 1981, Kvist et al 1982). Kvist et al (1982) found that Ii was present in the ER in amounts exceeding those of alpha and beta chains. As the Ia-Ii complex moves through the ER to the Golgi apparatus, it is terminally glycosylated at the 'high mannose' oligosaccharide units as well as at serine and threonine moieties to more complex forms (Owen et al 1981, Kvist et al 1982).

1.3.2.1.4 THE MHC CLASS II-ANTIGEN COMPLEX.

1.3.2.1.4.1 Structural Characteristics of the MHC Class II-Antigen Complex

The structural characteristics of peptides necessary for their interaction with MHC molecules has been an area of intensive investigation (Grey et al 1989, Sette et al 1989a, Kurata & Berzofsky 1990, Bhayani & Paterson 1989). The above workers used immunogenic peptides derived from chicken ovalbumin (OVA) 323-329 (Sette et al 1989a, 1989b) pigeon or moth cytochrome C 93-104 (in pigeon) (Bhayani & Paterson 1989) and sperm whale myoglobulin P133-146 (Grey et al 1989). As detailed above (Section 1.3.2.1.1) T-helper cells only recognise antigen when it is physically

altered (denatured or partially degraded) (Sette et al 1987) and subsequently re-expressed. The association between antigenic peptides and the MHC molecule has been controversial. The antigenic peptide and the MHC molecule could both bind to a receptor on the T cell, known as the T cell receptor (TCR) independently of one another, ie, dual recognition by the TCR. (Ploegh et al 1981, Ashwell & Schwartz 1986). The other alternative is that the T cell could serve as a site for the antigen and MHC molecule to critically interact with each other (Watts & McConnell 1986, Babbitt et al 1986).

Recent biochemical evidence supports the latter alternative, namely the concept of a specific receptor-ligand association (Watts & McConnell 1986, Buus et al 1986) to form a novel bimolecular structure which then serves as a substrate for recognition by T cells (Babbitt et al 1985). Moreover, it has been demonstrated experimentally using equilibrium dialysis that the MHC-peptide (receptor-ligand) interaction is the only absolute requirement for T cell recognition (Buus et al 1987). It is still unknown how the MHC molecules regulate the immune response during antigen presentation (Babbitt et al 1985) but it is thought to result from the stabilising effect of their association with the antigenic peptides.

It is known that T-helper cells only recognise antigen that is presented in association with MHC molecules (Rosenthal & Shevach 1973a).

1.3.2.2 FUNCTION OF MHC CLASS I

1.3.2.2.1 Endogenous Antigen Processing.

Initially from studies of target cell recognition by virus-specific cytotoxic T lymphocytes or CTL's (Braciale et al 1987), it was hypothesised that the MHC Class I-restricted lymphocytes recognise foreign antigens that are expressed in their native form on cell surfaces (Gething et al 1978). However, later reports illustrated that cleaved viral peptides were also capable of stimulating MHC Class I-restricted lymphocytes (Gaurti & Fan 1980, Maryanski et al 1986, Townsend et al 1985, 1988). The site on the human protein recognised by some Class I-restricted T cells is defined as a stretch of 16 amino acids (Bevan 1987). Whereas the complete native form seems irrelevant for restricted recognition (Bevan 1987). These findings have led to the hypothesis that processing of antigen for MHC Class I-restricted presentation, like that of MHC Class II, also appears to involve proteolysis and presentation of antigenic peptides bound to MHC Class I molecules to CTL's (Bjorkman et al 1987a, Townsend & McMichael 1987).

Morrison et al (1986) found that isolated influenza haemagglutinin (HA) polypeptide antigens that were recognised by Class I-restricted CTL's were not sensitive to lysosomotropic amines such as chloroquine thus suggesting that antigen presentation to MHC Class I-restricted virus-specific T lymphocytes occurs by a distinctly different pathway to MHC Class II-restricted antigen presentation. It became evident that MHC Class I molecules present peptides derived from exogenous or cytoplasmic proteins synthesised by the cell (Berzofsky et al 1988), whereas MHC Class II molecules generally present peptides derived from endogenous protein (Cresswell 1990). Townsend et al (1988) showed that modification of the N-terminal amino acid of the influenza virus nucleoprotein enhanced the nucleoprotein's rate of cytoplasmic degradation. This increased the lysis of cells expressing the viral antigen by MHC Class I-restricted CTL's (Townsend et al 1988). This supports the hypothesis that peptides presented by MHC Class I molecules undergo proteolysis in the cytoplasm and not in the lysosomal granules (Cresswell 1990).

It is proposed that once the viral peptides have been generated by proteolysis, they associate with the MHC Class I molecules in the endoplasmic reticulum (ER) or an associated compartment (Cresswell 1990, Yewdell & Bennink

1990). This supposition is supported by the observation that brefeldin A, which inhibits transport from the ER to the Golgi apparatus, blocks the presentation of cytoplasmically processed antigen thereby inhibiting Class-I restricted T cell recognition of the virally-infected cell (Yewdell & Bennink 1989, Nuchtern et al 1989). The molecular events involved in peptide binding by MHC Class I molecules are, at this stage, far from clear (Cresswell 1990). Evidence suggests though, that the antigenic structures recognised on target cells by $CD8^+$ CTL's are binary complexes formed by the association of peptide fragments of protein antigens with MHC Class I antigens (Cresswell 1990). In the normal course of events, the associated peptide-MHC complex leaves the ER and travels through the Golgi apparatus where it is expressed on the plasma membrane (Nuchtern et al 1989).

1.3.2.2.2 BIOSYNTHESIS OF MHC CLASS I GLYCOPROTEINS.
The biosynthetic mechanism of MHC Class I heavy chain (alpha 1) is detailed above in Section 1.3.2.1.3.1 and as stated is very similar to that of MHC Class II protein subunits (Cresswell et al 1987).

1.3.2.2.3 THE MHC CLASS I-ANTIGEN PEPTIDE COMPLEX.
1.3.2.2.3.1 Function of the Associated Invariant Chain, ß2m.
Insight into the role of ß2m in HLA expression was gained from the analysis of the Daudi cell line (Arce-Gomez et al

1978). This cell line was established from a Burkitt's lymphoma, and has consistently been typed as HLA-A,B-negative (Ploegh et al 1981). This cell line does not synthesise ß2m and no mRNA encoding for ß2m is produced either (Ploegh et al 1981). The lack of ß2m led to the hypothesis that this might cause the failure of HLA-A and HLA-B antigen expression on the cell surface (Ploegh et al 1981). Ploegh et al (1979) demonstrated by pulse/chase labelling experiments that the synthesis of the HLA-A and -B chains does occur in near normal amounts in Daudi cells in the absence of ß2m. However, there was a lack of expression of the heavy chain (domain alpha 1) at the cell surface indicating that ß2m is essential for expression of HLA-A and -B antigens at the cell surface in Daudi cells (Ploegh et al 1979, Seong et al 1988). Allen et al (1986) have shown that a murine cell line lacking the ß2m gene, and that was transfected with an H-$2D^b$ gene expressed the heavy chain on the cell surface. This implies that ß2m association with the heavy chain is not required for its expression on the cell surface (Allen et al 1986). Later, Perarnu et al (1990) reported that it is not the association of ß2m with the heavy chain that results in cell surface expression, but rather the way in which the chains fold is important for efficient intracellular transport of Class I molecules and their subsequent expression on the cell surface. The posttranslational

association of the heavy chain alpha1 with ß2m as followed by pulse-labelled experiments can be seen quite early in the ER (Krangel et al 1979). Association of alpha1 and ß2m is complete within 5-10 minutes after the completion of the heavy chain and is accompanied by substantial changes in the conformation of heavy chain (Seong et al 1988, Sege et al 1981). Association is required for the rapid processing of the high-mannose oligosaccharides on the heavy chain that occurs in the Golgi apparatus (Sege et al 1981).

1.3.2.2.4 FURTHER FUNCTIONS OF MHC CLASS I

MHC Class I antigens play a crucial role in the cellular immune response by functioning as the antigen-presenting molecules that enable CTLs to discriminate between foreign or nonself from self antigens (Bodmer 1972). Thus both MHC Class I and Class II molecules possess a common feature: they are involved in the recogniti of self and the subsequent transmission of a specific message from one cell subpopulation to another (Gopas et al 1989).

MHC Class I molecules have also been described as part of more general phenomena, involving aspects such as MHC associated cellular adhesion by T cell adhesion molecules or CAM (Staunton 1988), contact inhibition as suggested by Medawar in 1978 and demonstrated by Curtis & Rooney (1979)

in syngeneic epithelial cells of mice, interaction with hormones such as insulin in transgenic mice (Allison et al 1988) and interaction with viruses where the Class I molecule acts as a receptor for the virus (Inada and Mir 1984). Due et al (1986) showed that MHC Class I heavy chain can associate with the insulin receptor by displacement of the ß2m by the insulin receptor in mouse hepatocytes. Recently a 11-kDa chemotactic protein thymotaxin was identified and shown to be identical to ß2m (Dargemont et al 1989). (Chemotaxis is a migratory process of the activation of leukocytes or other cells by chemoattractants (Barrett 1988)). Besides its association with the MHC Class I heavy chain, free ß2m could also be involved in chemotactic activity in the cell (Dargemont et al 1989).

1.3.3. THE NATURE OF THE MHC-ANTIGEN COMPLEX.

1.3.3.1. Characteristics of the Recognition Structure on B Cells.

B cells are the class of lymphocytes responsible for generating an antibody response. The molecules on the B cells responsible for specific recognition of antigens are the different classes of antibodies that can be expressed either as cell-surface molecules acting as receptors, or in secreted forms serving a variety of purposes (Roitt 1989). These include the initiation of complement mediated killing

and the inactivation of viral particles by direct binding of the antibodies to the virally-infected cell (Roitt 1989).

1.3.3.2 CHARACTERISATION OF THE T CELL ANTIGEN RECEPTOR

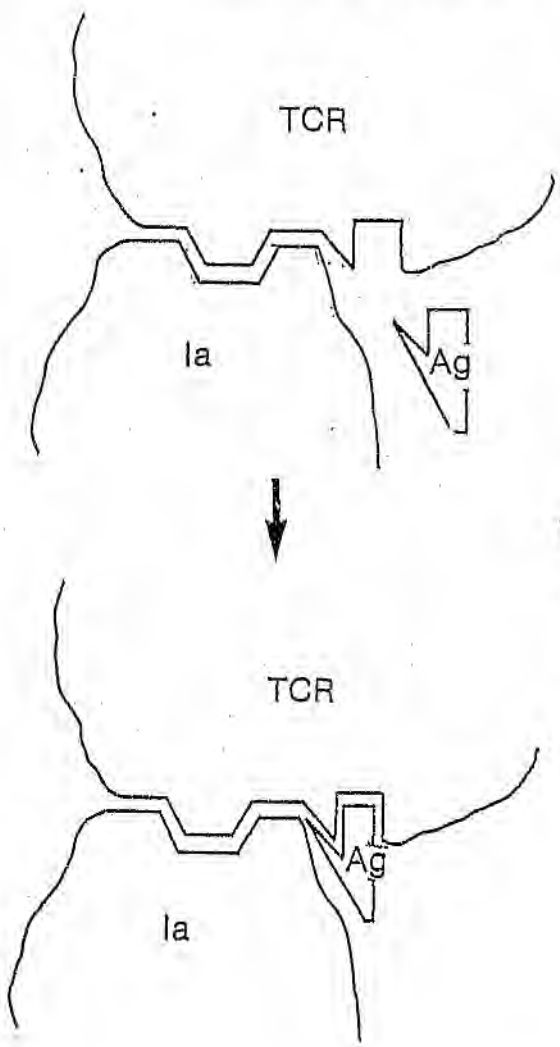
The equivalent recognition of the B cell's antibody structures on the T cell is the membrane-bound T cell antigen receptor or TCR (Davis & Bjorkman 1988). The TCR is specific for a combination of foreign antigen with an MHC molecule (Davis & Bjorkman 1988). The basic architecture of antibodies and their modes of interaction with antigen on B cells are well understood (Davis & Bjorkman 1988). By contrast, very little molecular data about TCR's or their ligands has been available until recently (Davis & Bjorkman 1988).

1.3.3.3 CHARACTERISTICS OF THE TCR WITH THE MHC-ANTIGEN COMPLEX

The characterisation of the antigen recognizing structure on the various classes of T cells has been a difficult problem for immunologists (Kappler et al 1981). Various models have been proposed to explain how T cells are able to demonstrate an apparent dual specificity for antigens and MHC products such that the T cell only functions when confronted with both the appropriate antigen and MHC products (Kappler et al 1981, Ploegh et al 1981). Some

workers proposed two independent TCR's (Ashwell & Schwartz 1986, Koch & Hämmerling 1982) while others have proposed a single TCR with a physical interaction occurring between antigen and MHC products on the antigen-presenting cell (Babbitt et al 1985, Shimonkewitz et al 1987, Benacerraf 1978, Buus et al 1986) in other words, the TCR recognises the antigenic peptide in association with the MHC molecule at a single site as described in section 1.3.2.1.4.1 (Davis & Bjorkman 1988). It was demonstrated by the use of an energy transfer of the TCR in an evanescent energy field that the MHC-peptide interaction is greatly stabilised by the TCR (Watts et al 1986). However, this hypothesis of a ternary or trimolecular complex (Sette et al 1989a, 1989b, Bhayani & Paterson 1989), between the peptide MHC-TCR was not demonstrated biochemically (Kappler et al 1981) until the elucidation of the three-dimensional structure of HLA-A2 by x-ray crystallography (Bjorkman et al 1987a, 1987b). (See Fig.1.4 for a proposed model of a ternary complex between TCR, MHC and antigen). The crystal structure illustrated that the MHC molecule interacts intimately with the antigenic peptide and identified the probable antigenic binding site to be a cleft on the outer surface of the MHC molecules between the two alpha-helices (Bjorkman et al 1987a, 1987b). Similarly, a hypothetical model of the antigenic binding site in the structurally similar MHC Class II molecules suggests that this class of

Figure 1.4 : The trimolecular complex for antigen specific T-cell receptor recognition of antigen and an Ia molecule (redrawn from Ashwell and Schwartz (1986))



TCR = T-cell receptor
Ag = Antigen
Ia = MHC class II

antigen contains a similar peptide binding region (Brown et al 1988). However, their hypothetical structure of the foreign antigen recognition site produces only a framework of generating testable models for peptide binding to MHC Class II. Berzofsky et al (1987), proposes that a single antigenic peptide associates with a MHC molecule in a specific conformation. This generates TCR diversity by overlapping epitopes (residues interacting with the TCR) being present on that specific molecular species (Berzofsky et al 1987). In order for the peptide to be immunogenic (Buus et al 1987) the epitopes must be predominantly amphipathic in nature (Berzofsky et al 1987), that is, having alpha-helices that have hydrophilic and hydrophobic residues on opposi' sides. Berzofsky's hypothesis may ultimately prove important in vaccine development for the prevention of AIDS (Acquired Immunodeficiency Syndrome) and Hepatitis B (Berzofsky et al 1987). It has recently been shown that a single peptide can bind in more than one way to a MHC molecule (Bhayani & Paterson 1989, Kurata & Berzofsky 1990). Not only may a peptide employ different agretopic residues (portion of the peptide interacting with the MHC molecule) (Berzofsky et al 1987) in binding to a MHC molecule but each peptide may interact with a MHC molecule in different conformations (Bhayani & Paterson 1989, Kurata & Berzofsky 1990). Thus a single MHC molecule appears to have different binding functions depending on

the TCR that make up the ternary complex (Bhayani & Paterson 1989, Kurata & Berzofsky 1990). This could perhaps explain the heretofore unknown phenomenon of how a specific MHC can interact with so many different antigens.

1.3.3.4 STRUCTURE OF THE T CELL RECEPTOR

There are four distinct TCR glycopeptides (alpha, beta, gamma, delta) that form two distinct disulfide-linked heterodimers (See Fig 1.2). Alpha:beta and gamma:delta are very similar to the immunoglobulins in primary amino acid sequence, gene organisation and tertiary structure (Davis & Bjorkman 1988, Sim et al 1984). Transfection of human cells with murine alpha:beta T-cell receptor genes established that alpha and beta chains of the TCR (Ti) are sufficient to transfer both antigen and MHC specificity to T cells (Sim et al 1984). In these experiments, cytotoxic T cells that bear T3 receptors were used (Sim et al 1984). It has also been established that antigenic peptides bound to the MHC molecules at what appears to be a single site is the major class of 'objects' that alpha:beta Ti cells 'see' (Bjorkman et al 1987b, Babbitt et al 1985, Davis & Bjorkman 1988). Yet another complicating feature of how a T cell sees antigen involves the CD4 and CD8 ("Cluster of Differentiation") glycoproteins found on the surfaces of T cells (Davis & Bjorkman 1988). CD4 or CD8 molecules, known

as 'accessory' molecules are only expressed on mature T cells (Gay et al (1988) The majority of $CD4^+$ cells are of the helper phenotype, while most $CD8^+$ T cells are cytotoxic or suppressor (Davis & Bjorkman 1988).

When discovered CD4 was named T4 or Leu-3 in man, L3T4 in mouse (Marrack et al 1983) and CD8 was named Leu-2 (Engleman et al 1981). Monoclonal antibodies against Leu-3 and Leu-2 were found to block T cell proliferation in response to Class II alloantigens in man and also block cellular cytotoxicity mediated by recognition of Class I molecules (Engleman et al 1981, Swain et al 1983, Biddison et al 1982). Marrack et al (1983) demonstrated that the L3T4 (CD4) molecule was not required for the functional activity of the TCR receptor with the antigen-MHC complex. However, later studies using gene transfer have illustrated that the CD4 and CD8 molecules markedly enhance T-cell responsiveness (Sleckman et al 1987).

CD4 is an invariant 55-kDa surface receptor (Littman 1987). Its expression on T lymphocytes is tightly associated with their restriction to MHC Class II antigens (Meuer et al 1984). Similiarly, the expression of CD8 correlates strictly with MHC Class I molecules. Gay et al suggested that both the TCR and CD4 molecules may bind to the same MHC molecule on the antigen presenting cell

(APC). Comodulation by TCR and CD4 suggests that CD4 may lie in close proximity to TCR (Gay et al 1988) or may even be associated with it (Chuck et al 1990). CD4-TCR complexes have recently been detected on the surface of human leukaemia T cells demonstrating that these complexes pre-exist on cell surfaces as stable molecular assemblies (Chuck et al 1990).

1.4. MHC EXPRESSION IN TISSUE

1.4.1. Tissue distribution of HLA-A,B,C Class I antigens

MHC Class I antigens (HLA-A, B, C) are expressed on the surfaces of all nucleated somatic cells (Daar et al 1984a, Fleming et al 1981, Barah et al 1970). Studies by Daar et al (1984a) of the detailed distribution of HLA Class I antigens throughout the body suggest that these antigens are not as ubiquitous in their distribution as previously suggested. Daar et al (1984a) suggests that the exaggeration of HLA Class I antigen distribution could be because techniques such as indirect immunofluorescence, do not allow detailed examination of tissue morphology. The use of monoclonal antibodies (MOABs) together with a sensitive immunoperoxidase staining technique as used by Daar et al (1984a) allowed for the examination of the detailed distribution of HLA Class I antigens.

HLA Class I antigens are expressed on virtually all adult tissues though at varying levels (Elliott et al 1989). The organ with the highest HLA antigen expression is the spleen followed by other lymphoid organs (Elliott et al 1989). The lowest amount of Class I expression occurs on muscle and nervous tissue (Elliott et al 1989) with neurones and skeletal muscle, under normal conditions, demonstrating no or weak Class I expression respectively (Daar et al 1984a). The primary distribution of "classical" HLA Class I antigens in the gut, glands, lung and kidneys is on epithelial derived cells (Elliott et al 1989). Vascular endothelial cells also express MHC Class I antigens (Elliott et al 1989).

1.4.2. DISTRIBUTION OF HLA CLASS I ANTIGENS IN LIVER

In studies of MHC Class I antigen distribution in normal tissue, it was found that all blood vessel endothelium, bile duct epithelium, fibroblasts, sinusoidal lining cells (including Kupffer cells) and interstitial dendritic cells expressed these antigens (Daar et al 1984a, Daar et al 1983, Barbatis et al 1981). However, the identification of Class I antigen on hepatocytes varied according to specimen used (Daar et al 1984a). Reports on the expression of Class I antigens by hepatocytes are conflicting. Several demonstrated no MHC Class I expression (Barbatis et al 1981, Lautenschlager et al 1984, Fukusato et al 1986)

whereas others reported distinct expression of HLA Class I antigen by hepatocytes (Montano et al 1982). The discrepancies of HLA Class I antigen expression on hepatocytes may be due to differences in the tissues used, specimen handling in processing, sensitivity of the method and interpretation of the staining as well as the inducement of HLA expression by anaesthetic agents or other drugs (Daar et al 1984a, Fukusato et al 1986).

However antigen display was noted on the cell surface of hepatocytes in many liver diseases including chronic active hepatitis (CAH), hepatitis B virus (HBV), hepatocellular carcinoma (HCC) among others as well as on certain hepatic cell lines (Fukasato et al 1986). Montano et al (1982) reported a relationship between the expression of HLA Class I antigens on hepatocytes and the level of HBV replication in chronic HBV infection: antigen expression on the hepatocyte plasma membrane is increased during the hepatitis E antibody (HBe Ab) phase of chronic infection. It is not presently known whether a common mechanism underlies the induction of HLA Class I antigens by such disparate factors as toxic agents, immunologic stimuli, viral infection and neoplastic transformation or whether the appearance of HLA Class I antigens represents a nonspecific manifestation of hepatocellular injury (Fukasato et al 1986).

1.4.3. TISSUE DISTRIBUTION OF HLA CLASS II ANTIGENS

In contrast to MHC Class I molecules, Class II antigens exhibit a more restricted tissue distribution (Elliott et al 1989). Initially, Class II antigens were described on B lymphocytes, epidermal Langerhans cells (Klareskog et al 1977) and endothelial cells (Hirschberg et al 1979). They were later found to be expressed on activated T cells (Fu et al 1978) as well as specialised antigen-presenting cells such as macrophages (Unanue 1981), dendritic cells (Hart & Fabre 1981) and Kupffer cells (Richman et al 1979) as well as fibroblasts (Boss & Strominger 1986). The results of a study by Daar et al (1984b) of the detailed tissue distribution of Class II antigens using a MOAB and the peroxidase-anti-peroxidase (PAP) staining technique on freshly frozen normal tissues, indicate that HLA Class II antigens have a far wider distribution than was previously thought. In normal human tissue, the distribution of HLA Class II antigens is found on the upper respiratory tract, small intestine, kidney and breast (in addition to above mentioned cells) (Daar et al 1984b).

1.4.4. DISTRIBUTION OF MHC CLASS II ANTIGEN IN LIVER

Studies on hepatocytes of normal liver and epithelial cells of the bile duct have demonstrated that these cells do not express Class II antigens (Daar et al 1984b). Other studies, however, have demonstrated that these antigens are

expressed by Kupffer cells (Walter & Israel 1987) and the interstitial dendritic cells around the bile ducts and portal triad (Daar et al 1983, Daar et al 1984b).

1.5 MHC EXPRESSION IN MALIGNANCY

1.5.1 Introduction

As first shown by Zinkernagel and Doherty (1979) T-cell mediated cytotoxicity of virally-infected and neoplastic cells require the presence of MHC Class I antigens. Foreign antigens (antigenic peptides) on the surface of accessory cells interact with the TCR and are identified as 'nonself' or abnormal only when presented in conjunction with 'self' Class I antigens (Lassam & Jay 1989). It appears that this interaction is essential for the removal of independent cell variants that presumably arise frequently in multicellular organisms (Freshney 1985). Thus the development of malignant tumours represents not only neoplasmic development but also the failure of host resistance to eliminate aberrant cells (Tanaka et al 1985). Transformation of a cell is alone insufficient to ensure the escape of tumour cells from immune surveillance (Tanaka et al 1985). It has therefore been hypothesised that suppression of MHC Class I antigens is a necessary step in the development and clonal selection of malignant cells (Katzav et al 1983, Hämmerling et al 1986) and is a possible mechanism whereby neoplastic cells escape from

immune surveillance (Tanaka et al 1985).

1.5.2. SUGGESTED MECHANISM OF MHC CLASS I SUPPRESSION

It has been suggested that suppression of the MHC Class I mRNA levels in a variety of highly oncogenic cells results from a substantial decrease in transcription initiation (Lassam & Jay 1989). This may explain how cancer cells can be made immunogenic by the derepression of endogenous Class I genes with modulating pharmacological or physiological agents such as the interferons or IFNs (Tanaka et al 1988), since class I mRNA accumulation is nearly always increased in IFN-treated cells (Tanaka et al 1988). It is currently believed that cis-acting DNA sequences both 5' and 3' to the transcription initiation site are required to achieve full induction (Korber et al 1987). Analysis of the 5' region of several HLA Class I genes has led to the identification of a 30 bp interferon response sequence (IRS) (Friedman & Stark 1985). Despite these advances in the characterization of the IFN response, it is still unclear whether IFN acts directly to induce Class I transcription or whether other mediators are involved (Tanaka et al 1988).

1.5.3. ROLE OF ONCOGENES IN MALIGNANCY

The search for the genes associated with oncogenesis has led to the identification of a group of highly conserved

genes whose products contribute directly to normal cell proliferation and differentiation as well as to the development of the organism (Bishop 1987). These cellular genes, termed proto-oncogenes or cellular oncogenes (c-oncs) may be converted to transforming genes by mutation and/or by molecular mechanisms (Carbone & Levine 1990). It has been found that certain oncogenes such as ras, which has been identified as a gene capable of inducing tumorigenicity (Sobel 1990, Barbacid 1987) as well as myc, mos, raf, src, fes and fms can induce metastatic potential in certain cancers upon transfection (Bishop 1987, Egan et al 1987). In addition it has been demonstrated that c-fos, a proto-oncogene that controls the expression of MHC genes may suppress metastasis by acting through a mechanism by which immunogenicity of the tumour cell is increased via expression of the H-2K antigen (Feldman & Eisenbach 1988).

1.5.4 ROLE OF MHC IN NATURAL KILLER CELL RECOGNITION

Natural killer or NK cells are responsible for the spontaneous lysis of a variety of neoplastic or virus-infected target cells (Heberman & Ortaldo 1981). Most of the evidence implicating NK cells as a defense mechanism against tumour growth and metastatic spread is based on the correlation between the susceptibility of tumour cells to lysis by NK cells in vitro and the

inhibition of the same tumour cells in vivo (Gopas et al 1989). It has been widely accepted that unlike T cells, natural killer cells recognise their targets in non-MHC-restricted manner (Gopas et al 1989). However, Kärre et al (1986) dispute this view and have proposed that NK cells are effector cells in an organism's defence system that are geared to detect the deleted or reduced expression of self-MHC. Kärre et al (1986) hypothesise that there is an inverse relationship between MHC Class I expression and susceptibility to lysis by NK cells. Some workers (Tanaka et al 1988) however, suggest that MHC antigens can clearly influence the susceptibility of certain tumours to NK lysis. However Péna et al (1990) recently demonstrated that HLA Class I antigens do not play a decisive role in NK susceptibility of cell lines derived from human solid tumours and suggested that molecules which are IFN-gamma inducible may confer NK susceptibility to these lines. Finally, Gopas et al (1989) suggested that there is no quantitative, inverse correlation between metastatic potential, NK activity and MHC expression. Studies on NK activity of lymphocytes isolated from patients suffering from HCC show a defective NK system (Son et al 1982, Saibara et al 1989). Although the cause of this depression is as yet unknown, it indicates a disturbed immune system in HCC and thus should be further investigated (Saibara et al 1989).

1.5.5 ANIMAL MODELS IN MALIGNANCY

Several experimental animal tumour systems have been used to provide evidence for the hypothesis that down-regulation of Class I antigens can allow transformed cells to escape immune detection (Lassam & Jay 1989). It has been shown by various workers that rodent cells that were transformed by the highly oncogenic strain of human adenovirus 12 (Ad-12) did not express MHC Class I antigens (Hämmerling et al 1986, Schrier et al 1983, Bernards et al 1983). However, in contrast, it was found that a non-oncogenic strain named Ad5 expressed normal levels of MHC Class I surface antigens (Schrier et al 1983, Bernards et al 1983). Transfection of a functional MHC Class I gene into a highly tumorigenic Ad-12 transformed murine cell line restored H-2K expression and resulted in a complete loss of oncogenicity (Tanaka et al 1985, Hämmerling et al 1986). This provided direct evidence that the oncogenic potential of Ad-12 transformed cells resulted from MHC Class I suppression (Tanaka et al 1985). Similar transfection studies with Class I-deficient cells derived from chemically-induced or spontaneous neoplasms have also demonstrated increased tumour rejection or reduced metastatic potential (Plaksin et al 1988, Wallich et al 1985, Hui et al 1984). Recently, differences in the regulatory functions of H-2D and H-2K gene products expressed on tumour cells have been identified (Gopas et al 1989). Feldman and Eisenbach (1988) and their co-workers

(Wallich et al 1985, Plaskin et al 1988) have established a relationship between metastatic potential and the ratio of H-2K to H-2D MHC molecules expressed on the cell surface (Eisenbach et al 1983). These workers have demonstrated, using metastatic clones of murine fibrosarcoma (Wallich et al 1985) or Lewis lung carcinoma (Plaskin et al 1988), that H-2 gene transfection resulted in conversion of a metastatic to a non- or low-metastatic phenotype. It appears that H-2D is involved in immunological tolerance, which is the failure of the immune system to reject the locally growing mass (Feldman & Eisenbach 1988). Thus H-2K has an immunogenic activity while that of H-2D causes suppression (Gopas et al 1989). Generally it appears that alterations of Class I antigens that affect recognition of tumour cells by CTLs can be because of:- a) quantitative regulation or loss of specific Class I antigens (Gopas et al 1989) b) aberrant expression of Class I molecules (Wortzel et al 1983) and c) generation of novel class I antigens. Aberrant Class I molecules have been detected on an ultraviolet radiation-induced fibrosarcoma 1591, a C_3H-derived tumour cell line (Wortzel et al 1983).

1.5.6 MHC REGULATION IN HUMAN CANCERS

In agreement with the animal models studies mentioned in Section 1.5.5, a reduction in MHC Class I antigen expression has also been observed in a variety of human

cancers. These cancers, particularly those of epithelial derivation, appear to express greatly reduced levels of surface MHC Class I antigens (Lassam & Jay 1989, Hui et al 1984). They include small-cell lung carcinoma (Doyle et al 1985), mammary carcinoma (Fleming et al 1981, Natali et al 1983, Möller et al 1989), and colorectal carcinoma (Momburg et al 1986, van den Ingh et al 1987, López-Nevot et al 1989 and Norazmi et al 1989) as well as gastric and laryngeal carcinomas (López-Nevot et al 1989). Similarly, cell lines derived from several of the above tumours also have reduced levels of MHC Class I antigen expression (Tanaka et al 1988). Natali et al (1989) hypothesise that the selective loss of a HLA polymorphic epitope such as HLA-A2 may explain the mechanism by which tumour cells of certain solid tumours escape immune surveillance.

1.6 HEPATOCELLULAR CARCINOMA

1.6.1 Introduction

Hepatocellular carcinoma (HCC), is one of the most common malignancies in the world (Wanebo et al 1989, Kew 1990). The prognosis of this tumour is so grave that its frequency rate and mortality rate are almost equal (Kew 1983). Reports have estimated it to be responsible for 250 000 to 1 250 000 deaths per year (Kew 1990, Cook & Moosa 1985, Chlebowski et al 1984). Its fulminant course, low rate of resectability and poor response to chemotherapy

make the prevention of this tumour of utmost importance (Kew 1983). Thus HCC presents one of the most urgent and important challenges in the field of liver disease (Kew 1990). Moreover, investigations of the many and diverse manifestations of this tumour may lead to a better understanding of the basic biology of neoplasia (Kew 1990).

1.6.2 EPIDEMIOLOGY

HCC occurs commonly age-adjusted incidences greater than 20 per 100 000 per annum, (Kew 1990)) throughout Africa south of the Sahara and in many parts of the Far East and Pacific Basin (Higginson 1963, Geddes et al 1970). The malignancy has a moderately high incidence (10-20 per 100 000 per annum) in Japan and Southern Europe (Dunham and Bailar 1968, Higginson 1963). HCC is uncommon or rare (2,9 per 100 000 men per annum) in North America, Britain, South America, Australia, India and in South African caucasians (Wanebo et al 1989, Kew 1990). The highest frequency of the tumour has been recorded in Moçambican Shangaans, who have an age-standardised incidence rate of 106.9 per 100 000 per annum (Prates & Torres 1965) although similar frequencies may exist in Chinese living in mainland China and Taiwan (Kew 1990). Recent evidence suggests that the frequency rates of HCC may be increasing in certain low incidence regions including North America and parts of Europe (Patton & Horn 1964, Saracci & Repetto 1980,

Manderson et al 1965). This increase may reflect a larger number of patients with alcoholic cirrhosis, improved treatment of this condition leading to a larger survival rate or the emergence of new etiological agents (Kew 1990).

The geographical distribution of the tumour is well defined even in countries of high incidences such as Moçambique in which the occurrence of the tumour is nine times more common in the eastern or coastal regions than in the inland areas (Harington et al 1975). Moreover, South African negroes have a higher incidence (28 per 100 000 men per annum, Kew 1990) than African-Americans (8 per 100 000 men per annum, Wanebo et al 1988) indicating that individuals of African or even Asian descent who migrate to countries with low incidences of HCC have lower incidences of the tumour compared with individuals remaining in their lands of origin (Higginson 1963). This phenomenon could be lifestyle-related (Kew 1990) and suggests that environmental factors rather than genetic or racial predisposition may play a role in the pathogenesis of HCC (Kew 1990). HCC occurs predominantly in men in all parts of the world (Kew 1990). In areas of high incidences the male to female ratio is 5:1 (Wanebo et al 1989) whereas in populations of low incidences there is a less striking preponderance of affected males (Kew 1990).

Worldwide the onset of the disease varies according to geographical location (Kew 1990) but it predominantly occurs in men over 30 years of age (Cook-Mozaffari and van Rensburg 1984). In South African negroes it is frequently encountered in their mid-twenties (Kew 1990) while in the Far East HCC generally develops at an older age with a mean age occurring at 52-59 years (Okada 1976). Although HCC is rare in children, hepatoblastoma is a distinctive tumour of the liver in infants and children from 2-6 years with most common age being three years (Shorter et al 1960).

1.6.3 ETIOLOGY

The pathogenesis of HCC is still unclear but available evidence suggests that the malignancy is multifactorial in etiology (Kew 1990, Wanebo et al 1989) and that the tumour has different causal factors in different parts of the world. Kew (1990) suggested that HCC exists in two forms. In areas of low incidence such as industrialised Western countries, the tumour occurs predominantly in older people being more common in males and is almost always a result of a complication of chronic cirrhosis. This type of tumour appears to have a better prognosis than that occurring in developing countries which have high incidences of HCC such as in sub-Saharan Africa and the Far East (Kew 1990). In these areas, the tumour occurs in younger men having little history of cirrhosis and has a

high rate of mortality Kew (1990). Kew has also suggested that different etiologic factors are operative in the two forms of the disease.

Even though racial and genetic factors appear to play some role in the pathogenesis of HCC certain risk factors such as environmental changes, aflatoxin ingestion, malnutrition, cirrhosis, chronic hepatitis B virus infection and as hormonal factors have been identified as the major etiological agents of the disease (Kew 1990, Wanebo et al 1989, Shar & Kew 1982).

1.6.3.1 HEPATITIS B VIRUS (HBV) INFECTION

The first evidence of an association of HCC with viral hepatitis was reported in West Africa by Paget in 1956 (Wanebo et al 1989). In 1970 Sherlock et al reported an association between chronic HBV infection and HCC. Studies of the etiologic relationship between HBV and HCC were advanced by the demonstration that a human tumour cell line PLC/PRF/5 has HBV DNA integrated into the cellular genome, which replicates hepatitis B surface antigen (HBsAg) (McNab et al 1976). The most compelling evidence incriminating chronic HBV infection in the pathogenesis of HCC worldwide came from Beasley's prospective study of 5500 HBV carriers and 19250 controls in Taiwan (Beasley et al 1981, Beasley 1988). His four year study established a relative risk of

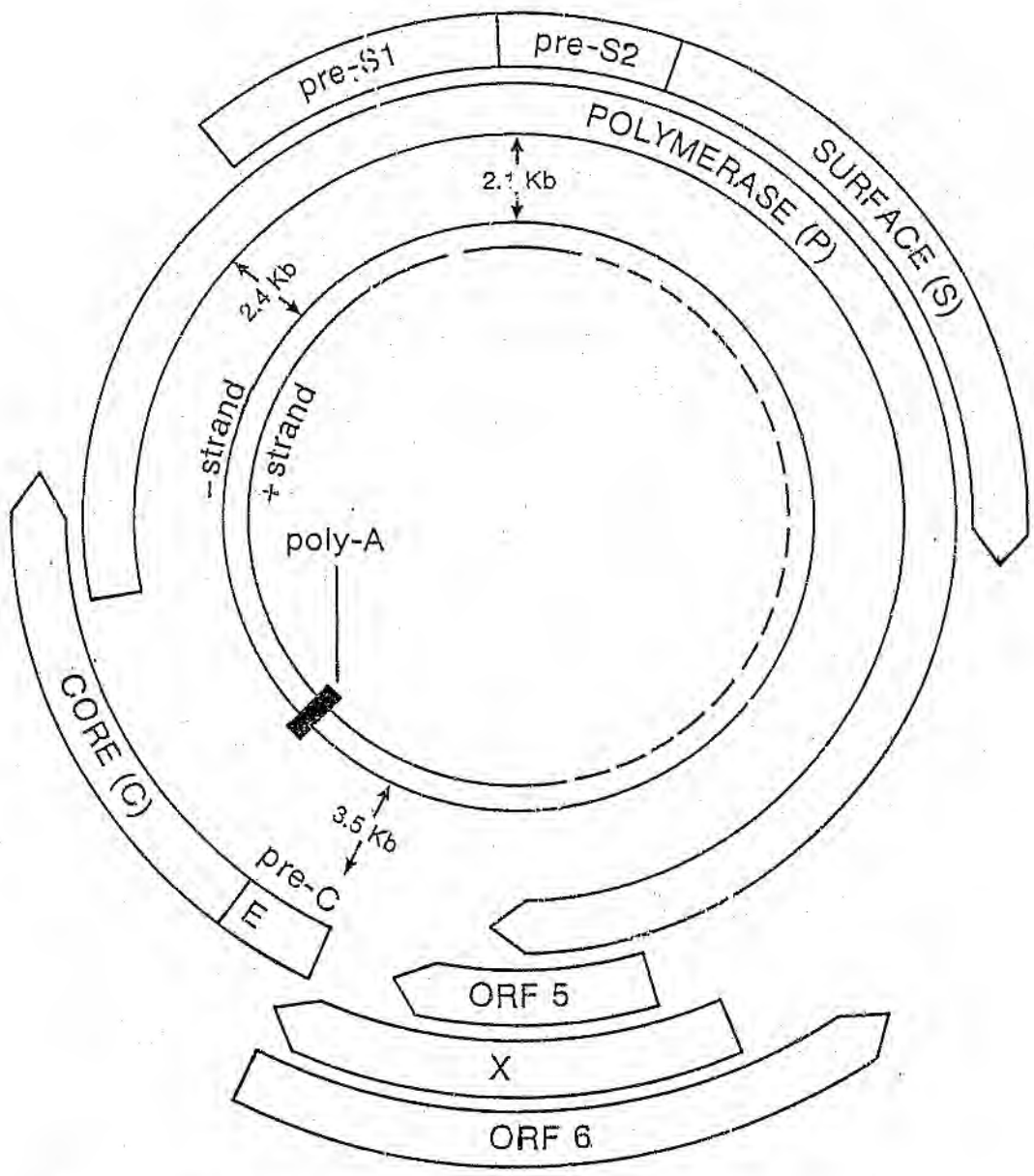
234 with HCC occurring in 40 carriers and 1 control subject (Beasley et al 1981). Further investigation has shown that there is a close geographical parallel between the existence of HCC and the prevalence of the HBV carrier state (Kew 1990). Kew et al (1986) have recently shown that in South African negroes when the HBV carrier rate decreases as a result of urbanisation the incidence of HCC shows a corresponding decrease. Studies have also indicated that development of HCC is not simply related to HBV infection but that persistent viral infection is required (Kew 1990). In areas of high incidence of the tumour, there are no exceptions to the correlation with a HBV carrier state, but in regions of low incidence such as Greenland and Chile there is the exception of relatively high HBV carrier rates and low incidences of HCC (Kew 1990).

The hepatitis DNA (hepadna) virus family currently comprises four members: human (Tiollais et al 1981); American woodchuck (Summers 1978); Beechey ground squirrel (Summers 1981, Marion et al 1980) and Pekin duck (Mason et al 1980). All the viruses are very similar and share unique features which define the virus family (Summers 1981). A characteristic feature of almost all active hepadna virus infections is the continuous presence in the blood of both noninfectious and infectious viral forms,

sometimes causing a silent infection in early life, which can lead to chronic infection (Robinson 1990).

The genome of hepadnaviruses are amongst the smallest of known viruses (Robsinson 1990). The HBV genome (See Fig 1.5) is a small circular, partially double-stranded DNA molecule with a single-stranded region of variable length (Summers et al 1975, Landers et al 1977, Hruska et al 1977).

Recent advances in gene cloning techniques have led to the detection of integrated HBV sequences in the cellular DNA of almost all HCC's studied (Shafritz & Kew 1981, Brechot et al 1980, Yaginuma et al 1985, Tur-Kaspa et al 1986, Dejean et al, 1986, Edman et al 1980). Two HBV sequences, the open reading frame (orf) x and the pre S2/S (See Fig 1.5) region have been implicated to play a causal role in liver carcinogenesis by trans-activation of cellular genes (Kekule et al 1990, Twu & Schloemer 1987). However not all HCC's appear to contain a functional orf x (Tur-kaspa et al 1986, Dejean et al 1986). The pre-S2/S is the only HBV gene (Fig 1.5) found to be integrated into almost all HBV-related HCC cases analysed so far (Brechot et al 1980, Edman et al 1980, Dejean et al 1986). Recently Kekule et al (1990) demonstrated that 3'-truncated pre-S2/S sequences in integrated HBV DNA of HCC's encode for an as yet



Map of the HBV genome

Figure 1.5: Redrawn from Robinson (1990)

unidentified transcriptional trans-activation activity.

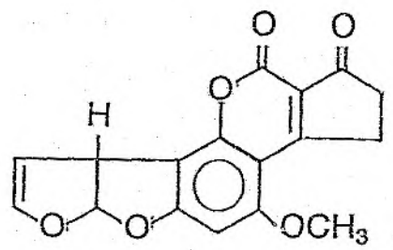
1.6.3.2 AFLATOXINS

There is no unequivocal evidence at present that any single chemical agent is responsible for causing HCC (Kew 1990). However, field studies have demonstrated a positive correlation between the incidence of HCC and the aflatoxin content of food eaten in various parts of Africa (Peers et al 1976, van Rensburg et al 1974), with the greatest intake of aflatoxins occurring in Moçambique (Kew 1990).
Aflatoxin is a mycotoxin that is a metabolite of the fungus Aspergillus flavus (A.flavus) (Kew 1990).
Aspergillus flavus is ubiquitous in nature and contaminates many staple foodstuffs including grains, soya, cottonseed and ground nuts especially in tropical climates where storage conditions favour the growth of the fungi (Linsell & Peers 1977). Aflatoxin B_1 (see Fig 1.6) is the most potent experimental hepatocarcinogen known with dosages of a few parts per billion inducing liver cancers in susceptible animals (Kew 1990). It is hypothesised that aflatoxin B_1, may play an etiologic role in the development of HCC acting either independantly or in combination with HBV (Kew 1990). Recently it has been suggested that mutations in the tumour-suppressor gene p53 are caused by aflatoxin B_1, or HBV, contributing to the high incidence of HCC in areas where these carcinogens are

major etiological agents (Hsu et al 1991, Bressac et al 1991). Kew (1990) proposes that even if aflatoxin is shown to be pathogenetically important in developing countries, it is unlikely that it plays a significant etiologic role in developed or Western countries.

1.6.3.3 RELATIONSHIP TO CIRRHOSIS

In 1901, Eggel recognised an association between HCC and cirrhosis. Since then numerous pertinent reviews have been published (Cameron 1976; Shikata 1976; Anthony 1979; Kew 1983; Zakim et al 1990) with cirrhosis itself being regarded as a preneoplastic state that often predisposes to hepatocarcinogenesis (Melato et al 1989). Because of these studies, differences in the relationship of cirrhosis and HCC as well as in the nature of the underlying cirrhosis have emerged. In 1983, Kew observed that in areas of low incidence (USA, UK and Canada) the tumour develops as a complication of a long-standing symptomatic cirrhosis whereas in areas of high incidence (Sub-Saharan, South East Asia) the two conditions frequently manifest simultaneously with death due to the tumour before the onset of symptoms and signs of cirrhosis. In 1976, Shikata described the type of underlying cirrhosis in countries with a low incidence as being predominantly micronodular with a macronodular form being more common in Africa and the Far East. Becker and Chatgiadakis (1961) noted that



Aflatoxin B_1 - **chemical structure**

Figure 1.6: Redrawn from Kew (1990)

South African Europeans manifest micronodular cirrhosis compared with the macronodular form observed in South African negroes.

Generally, the micronodular form is associated with cirrhosis due to its most commonly recognised aetiologic agent, alcohol, while that of the macronodular variant is associated with viral or toxic factors (post-necrotic or post-hepatic) (Anthony 1979). Cirrhosis comprising nodules of less than 3 mm in diameter are classified as micro-nodular whilst those larger are macronodular (Anthony et al 1978). The incidence of cirrhosis in cases of HCC, when internationally reviewed, varies from between 60 and 90 percent which may be due to the different forms of cirrhosis in varying geographical locations (Szmuness 1978).

The role of alcohol in hepatocarcinogenesis is still uncertain (Anthony 1979) although it has been reported that the presence of cirrhosis rather than the direct effect of alcohol is more important in the carcinogenic process (MacSween 1982). Mechanisms other than the production of cirrhosis whereby alcohol could take part in the carcinogenic process have also been reported (Lieber et al 1979; MacSween 1982). Although alcohol has not been shown to act as a carcinogen in the experimental situation, it

may act as a co-carcinogen by provoking cell proliferation or by enzyme induction within the liver, resulting in the metabolism of previously inert carcinogenic factors (Alan Paterson-PhD thesis). The possibility that malnutrition accompanying alcoholism may play a role in carcinogenesis has also been considered (Lieber et al 1979). It has been established that known carcinogens e.g. nitrosamines, can contaminate some alcoholic beverages which may contribute to hepatocarcinogenesis (MacSween 1982). Furthermore, there is now evidence that HBV infection may overlap with alcoholic liver damage and that this may be a factor in liver carcinogenesis (Brechot et al 1982, Brechot et al 1985). Mills et al (1979), investigating the incidence of the antibody to HBsAg (anti-HBs) in chronic liver disease (CLD) observed a significantly increased frequency of the antibody in alcoholic liver disease as compared with other causes of CLD and in controls. Other studies have supported this finding (Chopra et al 1980, Goudeau et al 1981) while Bassendine et al (1983) were unable to demonstrate a similar trend in their study's cohort. However, Brechot et al (1982), in a study on alcoholic livers using routine serology and recombinant DNA techniques, demonstrated that patients suffering from both alcoholic cirrhosis and HCC all had HBV integrated into the tumour cell genome. Of the cases of alcoholic liver disease, 37 percent were serologically positive for HBV

with 3 out of 51 displaying HBV-DNA integration into the host genome (Brechot et al 1982). On the basis of these data the authors suggested that HBV plays a part in the development of HCC in the presence of alcoholic cirrhosis.

1.6.3.4 HORMONAL AND STEROID-RELATED FACTORS

Androgenic steroids have become an acknowledged risk factor in the development of HCC since an association was first described by Johnson et al (1972). The role of oral contraceptives and oestrogens is somewhat more controversial. Initially the relationship appeared to be with benign adenomas (Klatskin 1977; Ishak 1981) but in recent years there have been numerous but contradicting reports of HCC developing in women taking contraceptive steroids. (Kew et al 1990).

1.7. MHC EXPRESSION IN HEPATOCELLULAR CARCINOMA

As detailed above (Section 1.4), neither Class I nor Class II MHC antigens are detectable on the surface of normal hepatocytes however both classes of MHC antigens are displayed in various pathologies including hepatitis. These findings suggest that expression of MHC antigens can be induced under certain circumstances.

In the study of HLA expression in HCC, Paterson et al (1988) reported a homogenous enhancement or acquisition of

MHC Class I antigens in the majority (94,3%) of tumour specimens with a concomitant expression of the HLA Class I-associated invariant chain, β2m, in all but 2 cases. MHC Class II expression was observed in approximately half of the specimens examined, (Paterson et al 1988) PAP staining in conjunction with monoclonal antibodies (MOABs) resulted in a sparse and heterogenous display. Whereas, the invariant chain (Ii) also present in approximately half of the cases, was frequently intensely stained and extensive in distribution (Paterson et al 1988).

Several permanent human hepatocellular carcinoma (HCC) cell lines have recently become available. These cell lines are useful tools for the study of MHC gene expression and regulation in hepatocytes as well as for the investigation of the association of MHC antigens with transformation of human hepatocytes (Sung et al 1989). HCC cell lines also present useful models for investigating the association of MHC antigens with hepatocyte differentiation and transformation, and for explaining the role of these antigens in host immunity to HCC (Sung et al 1989).

The present study therefore employed the use of HCC cell lines as a model to further the understanding of MHC antigen expression in this carcinoma. The first aim was to elucidate the expression of MHC antigens on the cell lines under spontaneous conditions. These experiments were

performed in order to establish a control or baseline level of MHC antigen expression for use in further experiments in this study.

The second aim was the investigation and elucidation of the effect of potential modulating agents on antigen expression. An increase in MHC antigen expression in HCC cell lines using certain agents has been reported by other workers (Sung et al 1989, Krief et al 1988, Fukusato et al 1988, Yoshioka et al 1989). Such an increase in expression may suggest a potential therapeutic value of these modulating agents as well as further clarifying the biologic significance of the aberrant MHC expression observed in HCC.

CHAPTER 2

CELL CULTURE

2.1 Introduction

Tissue culture was devised at the beginning of this century (Carrel 1912) as a method for studying the behaviour of animal cells free of system variations that might arise in the animal both during normal homeostasis and under the stress of an experiment (Freshney 1987). In 1907 Harrison chose the frog as his tissue source, presumably because it was a cold-blooded animal, and therefore incubation was not required. Later, the embryonated hen's egg, which provided diversity of cell types in primary culture was used (Freshney 1987). In 1943 Earle *et al* produced the first continuous mammalian cell line using rodent tissue. In 1952 Gey *et al* demonstrated, with the establishment of the HeLa line derived from squamous carcinoma of the cervix, that human tumours could also give rise to continuous cell lines. Since then most of the interest in tissue culture has remained in avian and mammalian tissue (Freshney 1987). The term cell culture was coined to refer to cultures derived from dispersed cells taken from the original tissue by enzymatic, mechanical or chemical disaggregation and today the terms cell culture and tissue culture are interchangeable (Freshney 1987). The development of cell

culture as a modern, sophisticated technique has been helped by technical improvements such as the commercial supply of reliable media and sera, greater control of contamination with antibiotics and clean air equipment making it accessible to a wide range of interests (Freshney 1987). Cancer research ranging from understanding neoplasia to the study of cell interactions and intracellular control mechanisms in cell differentiation (Freshney 1985), metastasis (Feldman & Eisenbach 1988) and tumourigenicity (Tanaka et al 1985); chromosomal analysis of cells derived from the womb by amniocentesis (Valenti 1973), monoclonal antibody production by hybridoma techniques (Köhler & Milstein 1975), somatic cell genetics studies using somatic cell fusion techniques (Barski et al 1961, Littlefield 1964) and genetic analysis of inherited traits (Buchwald 1984) are among some of the areas of research that depend heavily on tissue culture techniques.

Besides the abovementioned advances, growing cells in culture has at least three major advantages (Freshney 1987, Buchwald 1984). Firstly, the precise control of the physiochemical environment (pH, temperature, osmotic pressure, O_2 and CO_2 tension). Secondly, the control of the physiological conditions where the cells can be isolated from the biological milieu of the donor and kept

under relatively controlled (but not always defined) conditions (Freshney 1987, Buchwald 1984) is possible. The third advantage is that cell cultures can make each donor immortal since culture may produce a homogeneous cell type which can be perpetuated over several generations or indefinitely stored in liquid nitrogen in medium containing a cryoprotective agent, for example glycerol or DMSO (Freshney 1987, Buchwald 1984). The major disadvantages of cell culture are the strict aseptic conditions and expertise of the operator required (Buchwald 1984). Besides contamination by bacteria, moulds and yeasts, contamination with micro-organisms such as mycoplasma from the media used or from the operator result in the culture being compromised (Freshney 1987, Buchwald 1984). Another disadvantage is the expenditure of effort and materials required to maintain relatively little tissue with the cost of producing cells in culture being about ten times that of animal tissue (Freshney 1987). Furthermore, many continuous cell lines exhibit variability from one passage to the next. (Freshney 1987, Buchwald 1984). Thus the validity of the cultured cell as a model of physiological function *in vivo* has been frequently criticised (Freshney 1987). The most obvious difference of cell culture to the *in vivo* situation is the alteration of the cellular environment, caused by the dissociation of cells from a three-dimensional geometry to their

propagation on a two-dimensional substrate. In addition there are changes in cellular metabolism such as energy metabolism and production of hormones. The culture environment also lacks several systemic components (the endocrine and nervous systems) essential for homeostatic regulation **in vivo** (Freshney 1987).

Although, the existence of such differences cannot be denied, many specialised functions are expressed in culture such as alpha fetoprotein (AFP) production in the HCC cell line Hep 3B as reported by Knowles (et al 1980) (Freshney 1987). Thus, appreciating the limits of the model, cell culture can still be a very valuable tool (Freshney 1987) for analysing **in vivo** situations.

The process of cells undergoing malignant or neoplastic transformation **in vitro** is termed the evolution of cell lines and the point of transformation a 'crisis' (Freshney 1987). After evolving the cell line becomes continuous, immortalized (Freshny 1987). Since malignancy cannot be easily demonstrated **in vitro**, properties pertaining to malignant cells **in vivo** are applied to the **in vitro** situation (Freshney 1987). The models testing neoplastic properties such as tumourigenesis, invasiveness and metastatis **in vitro** have mostly been demonstrated by the use of a similar experimental approach. Tumour cells

that are grown in vitro are injected into laboratory animals and the resulting neoplastic process, if any, observed (Tanaka et al 1985, Feldman & Eisenbach 1988).

2.2 CULTURE OF HEPATOCYTES

Establishment of normal adult cells in culture is exceedingly difficult but advances in cell culture technology have only resulted in the maintenance of some normal or months in culture (Reid & Jefferson 1984).

Chang established the first normal hepatocyte cell line in 1954 (Chang 1954) and since then many attempts have been made to isolate and culture human hepatocytes (Maekubo et al 1982, Hsu et al 1985, Reid & Anderson 1984, Demoise et al 1971, Reese & Byard 1981). Various methods have been attempted with viable hepatocytes being successfully isolated by perfusion of the liver tissue specimen with collegenase (Hsu et al 1985, Ballet et al 1984) using conditions based on those optimised in the pig (Ballet et al 1984). Digestion with the protease collegenase coupled with other enzymes that digest structural protein such as dispase (Hsu et al 1985) or hyaluronidase (Bellemann et al 1977) either after perfusion (Ballet et al 1984) or as a nonperfusion technique (Bellemann et al 1977) also proved to be a successful method for obtaining viable hepatocytes. Co-culturing of hepatocytes with fibroblasts

as feeder layers was demonstrated to improve hepatocyte function and lifespan in culture (Reid & Anderson 1984). Ultrastructural and biochemical examination of the hepatocytes in culture showed resemblance to many characteristics of the liver (Bellemann et al 1977; Ballet et al 1984). However, in general, after hepatocytes were plated onto cell culture plastic into a serum-supplemented medium, they changed into a squamous-like shape, exhibited rapid biochemical and morphological deterioration and within two weeks had lost their viability (Reid & Jefferson 1984).

2.3 HUMAN HCC CELL LINES

The establishment in vitro of cell lines derived from HCC is a logical approach to the laboratory investigations of the tumour. Permanent cell lines derived from these tumours allow for a range of studies that cannot be carried out on biopsy or postmortem material (Alexander et al 1976). The majority of hepatoma cell lines were initially derived from rodent hepatocytes (Darlington 1987). One of the first human HCC cell lines described was designated by Chen in 1964 as (Chen 1964). In 1975, Fogh & Trempe established the line SK Hep 1 from an ascites effusion of an adenocarcinoma of the liver (Alexander et al 1976), although this line is subsequently considered to be fibroblastic in nature (Alexander-personal communication). In 1976 Doi established two cell lines from a

hepatoblastoma that were morphologically distinct but both exhibited AFP production (Doi 1976). Also in 1976, Alexander et al established a HCC cell line from a primary hepatoma of an African male and designated it PLC/PRF/5 (Alexander 1976). This cell line exhibits a variety of hepatospecific functions as well as AFP and albumin production (Darlington 1987). It has been studied with respect to the integration of the HBV genome in the cellular DNA and its expression of the HBV surface antigen (HBsAg) (de Koning et al 1984, Knowles et al 1980, McNab et al 1976).

Aden and co-workers established two lines Hep 3B and Hep G2 from primary hepatocellular carcinoma and hepatoblastoma respectively (Knowles et al 1980, Aden et al 1979). Their method utilised mouse feeder layers (Aden et al 1979). Hep 3B, a well differentiated cell line, has been characterized with respect to the production of albumin, AFP, HBsAg as well as other liver-specific phenotypes (Darlington 1987). The expression of differentiated functions of Hep G2 has also been extensively studied (Aden et al 1979, Bouma et al 1989, Darlington et al 1987). Although HBsAg or integration of viral DNA are absent in this cell line, HepG2 has retained a large number of hepatospecific phenotypes (Darlington 1987). Chang et al (1983) established the poorly differentiated HA22T/VGH cell line

from a Chinese male with HCC. This cell line does not express HBsAg (Chang et al 1983). Unlike all of the above cell lines which only grow in monolayers, HA22T/VGH can be cultivated not only in a monolayer, where it expresses some hepatospecific gene products (such as C3 and C4 complement) but also as suspended cell aggregates where its production of liver-specific products as well as plasminogen and albumin increases (Chang et al 1983).

Mahlavu was successfully established from a needle aspirate from the edge of a suspected tumour nodule (Prozesky et al 1973). This cell line expresses HBsAg and appears as polygonal epitheloid cells which tend to grow in sheets (Prozesky et al 1973).

Tong PHC was established from an Italian male with primary HCC (Lin et al 1984). This cell line secretes both HBsAg and AFP into the culture supernatant and several HBV-DNA integration sites have been demonstrated (Lin et al 1984).

All of the above cell lines can be maintained in defined media such as DMEM or RPMI-1640 supplemented with 10% heat-inactivated FCS (personal communication Dr E Bey, Highveld Biologicals, Jhb. SA).

Various studies testing the comparative morphology and

tumourigenicity of HCC cell lines have been performed (Shouval et al 1988). These studies have attempted to produce tumours in athymic rats and mice as a method of establishing whether the cultured cells are indeed malignant and not from non-malignant components of the original tumour specimen (Fogh et al 1977).

Shouval et al (1988), investigating the cell line PLC/PRF/5 Hep G2 and Mahlavu, found that when the cell lines were inoculated subcutaneously (s.c.) into athymic Balb/c nude mice and N/NIH outbred nude rats, well-encapsulated tumours were produced. In nude rats, PLC/PRF/5 cells were the most tumourigenic followed by Mahlavu Experiments using Hep G2 were not performed in nude rats since these cells were extremely difficult to grow in athymic mice (Shouval et al 1988). In athymic mice, PLC/PRF/5 was the most tumourigenic followed by Mahlavu and then Hep G2 (Shouval et al 1988). Metastases were infrequently observed following s.c. hepatoma cell injection, regardless of HCC cell type used and irrespective of animal strain (Shouval et al 1988). This correlates with the in vivo situation, in which pulmonary metastases are generally confined to terminal cases (Kew & Paterson 1985). The above types of experiment confirm the malignant phenotype of the HCC cell lines as well as providing a model for assessing the response of

novel modes of treatment for human HCC.

Because the study of the characteristics of human hepatoma cells is limited by the availability of tissue, HCC cell lines provide a model for studying the differentiation of human hepatocytes (Chang et al 1983). Since HCC cell lines exhibit various hepatospecific phenotypes (Darlington 1987) they can be studied for their expression and regulation of hepatocyte markers as well as the induction of hepatocyte markers in vitro. It was therefore of interest to examine the expression of the major histocompatibility gene products on the surface of such cell lines in the present study.

2.4. MATERIALS AND METHODS

2.4.1. Cell lines

Cell lines used are listed in Table 2.1 and were obtained from Highveld Biologicals, Johannesburg, SA and as kind gifts from Dr S Aspinall (Medunsa, GaRankuwa) to Professor A Paterson. On examination they were found to be free of a mycoplasmal contamination. The absence of mycoplasma was confirmed by Hoechst DAPI fluorescent stain (Highveld Biologicals).

2.4.2. DEFINED MEDIA USED

Dulbecco's modification of Eagles MEM (DMEM) (Dulbecco and

Freeman 1959, Morton 1970) and RPMI-1640 (Moore et al 1967) were obtained commercially (Highveld Biologicals, Johannesburg, SA). HCC cell lines were grown in DMEM and cells in suspension in RPMI-1640 medium. Foetal calf serum (FCS) was used at a concentration of ten and five percent for HCC cell lines and cells grown in suspension respectively. Appendix 3 describes in detail the constituents of both RPMI-1640 and DMEM.

2.4.3. MAINTENANCE OF CELL LINES

Cultures were grown in disposable 25 and 75 cm^3 cell culture flasks (Cel-Cult, Sterilin, Hounslow, UK) in a humidified 5%CO_2-95%air incubator at 37°C. The growth of the culture was monitored daily and cultures fed approximately three times per week with fresh medium. Cells from confluent cultures were trypsinized from their substrate (approximate time to reach confluency being one week) using a freshly made trypsin (0,25%)/EDTA (0,01%) mixture (both from Highveld Biologicals, Johannesburg, SA) at a ratio of 1:1 at 37°C and disaggregated into a single cell suspension. Trypsinization was terminated by the addition of fresh medium containing 10% FCS at 37°C. The cells were harvested by centrifugation for 5 min at 200 g. Cells were resuspended in 5ml fresh medium at 37°C and aliquots withdrawn for counting on a hemacytometer and viability assessment by the trypan blue dye exclusion test

using standard procedures. Cells grown in monolayers that were used for experiments had a viability of 85-90% while cells grown in suspension that were used for experiments had a viability of greater than 95%. Thereafter, cells were diluted further in fresh medium and subcultured by plating them out in cell culture flasks. Cells grown in suspension were harvested by centrifugation for 7 min at 200g, counted and their viability assessed. Cells were then further diluted in fresh medium supplemented with 5% FCS (Appendix 3) and incubated in a humidified 95% air- 5% CO_2 incubator at 37°C.

2.4.4 PRESERVATION OF CELLS

Cells were preserved at a cell concentration of 10×10^6 cells/ml using glycerol as a cryoprotective agent (Appendix 3) and stored in liquid nitrogen for up to six months without adverse effects on their viability (Rijntjies et al 1986). When needed, cells were thawed (Appendix 3), counted, their viability assessed and reseeded.

TABLE 2.1 LIST OF CELL LINES USED IN THIS STUDY

HCC Cell lines	Appearance	Reference
PLC\PRF\5	Epithelial-like	Alexander et al (1976)
Hep G2	"	Aden et al (1979)
Hep 3B	"	Aden et al (1979)
Tong PHC	"	Lin et al (1984)
HA22T/VGH	Fibroblast-like	Chang et al (1983)
HA59T/VGH	"	Kind gift of C Chang
Mahlavu	"	Prozesky et al (1973)
Control Cell Lines		
Raji[a]	Spherical cells in suspension	Pulvertaft (1965)
K562[b]	"	Lozzio & Lozzio (1975)

a. Raji was established in 1965 from the tumour tissue of a Burkitt's lymphoma (Pulvertaft 1965). It has been typed to express MHC Class I and II antigens as well as their respective invariant chains (Nilsson et al 1974)

b. K562 was originally established from a pleural effusion of a patient with chronic myelogenous leukemia in terminal blast crisis.

2.5 RESULTS AND DISCUSSION

Cell lines were successfully grown according to suppliers' recommendations. The appearance of the various cell lines is summarised in Table 2.1. The cell lines PLC/PRF/5, Hep 3B, Hep G2 and TONG PHC appeared similar on morphological examination under a microscope. All were epithelial-like and grew in monolayers. The Alexander cell line PLC/PRF/5, appeared under microscopal examination, to be squamous-like with canaliculi and sinusoid-like formations sometimes visible. In the cell line Hep G2 after reseeding, the cells appeared to grow from islands with the islands eventually forming a monolayer (Aden et al 1979). Hep 3B appeared under the microscope as squamous-like cells with monolayer growth evident. HA22T/VGH, HA59T/VGH and Mahlavu appeared as more fibroblast-like with a monolayer cell growth. Mahlavu tended to grow in sheet-like fashion. K562, an erythromyeloid cell line (Nilsson et al 1974) was spherical in shape with projections that of MHC antigens (Lozzio & Lozzio 1975) were refractile under phase microscopy. It tended to form clumps of cells in suspension during growth periods, which were easily disaggregated on gentle shaking. Raji, a B-lymphoid derived cell line, grew as smooth spherical cells in suspension.

The abovementioned HCC and control (Raji and K562) cell lines were used for all experimental work performed during this present study.

CHAPTER 3

INDIRECT IMMUNOFLUORESCENCE ASSAY

3.1 Introduction

Immunofluorescence is widely used for the study of cell surface antigens (Boucheix et al 1985). Compared with the radioimmunoassay (RIA) or the enzyme linked immunosorbent assay (ELISA) it allows for study at the single cell level (Boucheix et al 1985). The use of indirect immunofluorescence assay is hampered by the fact that it is difficult to perform quantitative studies, however it ensures rapid qualitative results (Boucheix et al 1985). The use of a specific conjugated antibody such as fluorescein isothiocyanate (FITC) depends on the fact that dye molecules can be chemically linked to antibody molecules without impairing the capacity of the antibody to react specifically with the antigen (Marrack 1934). Considering these favourable aspects and because of its simplicity and ease of experimentation, the expression of MHC antigens on hepatoma cells grown in culture was assessed.

3.2 MATERIALS

3.2.1 Primary Antibodies

i) W6/32. (Dakopatts, Denmark) A mouse

anti-human monoclonal antibody directed against the monomorphic determinants of HLA-A,B,C (Parham et al 1979)

ii) TAL IB5 (A kind gift of J. Bodmer, ICRF, UK) A mouse anti-human monoclonal antibody directed against the heavy, nonpolymorphic chain of HLA-DR (DR-alpha) (Adams et al 1983).

iii) Anti-Beta-2-[illegible] (anti-ß2m) (Dakopatts, Denm[illegible] anti-human monoclonal antibody d[illegible] the HLA Class I-associated invarian[illegible] ß2m (Bjerrum & Birgens 1986).

iv) VIC Y1 (An-Der-Grub Bioresearch, Kaumberg, Austria). A mouse anti-human monoclonal antibody directed against the HLA-D region-associated invariant chain, Ii (Antibody isotope IgG_1) (Quaranta et al 1984).

Table 3.1. summarises the various primary antibodies and their specificities used in the current study.

3.2.2 SECONDARY ANTIBODY

Flourescein Isothiocyanate (FITC) conjugated F $(ab^1)_2$ fragment of rabbit anti-mouse

TABLE 3.1. LIST OF ANTIGENS AND PRIMARY ANTIBODIES AND THEIR SPECIFICITIES.

Clone	Specificity	Source	Reference
W6/32	HLA-A,B,C	Dakopatts, Denmark	Parham *et al* (1979)
TAL IB5	HLA-DR	Gift of J Bodmer (ICRF, UK)	Adams *et al* (1983)
anti-ß2m	ß2m	Dakopatts, Denmark	Bjerrum & Birgens (1986)
VIC Y1	Ii	An-der-Grub Bioresearch Kaumberg, Austria	Quaranta *et al* (1984)

immunoglobulins. The antibody reacts with all mouse IgG subclasses, and mouse IgM and IgA (Dakopatts, Denmark).

3.2.3 CONTROL ANTIBODY

A FITC-conjugated goat anti-serum to human immunoglobulins (IgG, IgM, IgA) (Hyland, USA) was used as a non-specific antibody.

3.3. METHODS

The indirect immunofluorescence assay (IIF) was performed on viable hepatoma cells grown in monolayers using a modification of the protocols described by Pernis and Tse (1985). In order to examine the effect (if any) of fixing on MHC expression and to assess which method was optimal experimentally, modified techniques for assessing immunofluorescence on both viable and fixed cells were adopted for the control cell lines K562 and Raji.

3.3.1 INDIRECT IMMUNOFLUORESCENCE ASSAY

The techniques of Pernis and Tse (1985), modified for indirect immunofluorescence (IIF) staining of hepatoma cells, were performed as described below. Confluent monolayer HCC cells were trypsinized, counted on a hemacytometer and their viability assessed by a trypan blue dye exclusion test as described in section 2.4.3. The

single cell suspension was diluted to approximately $2x10^4$ cells/ml in complete DMEM (Section 2.4.2). Cells were cultured on 22x22mm glass coverslips (Mentzel-Glaser, Germany) in plastic petri-dishes (Promex, South Africa) in an humidified incubator containing 95% air-5% CO_2 at 37°C. After 24 hours the viable cells had adhered only to the surface of the glass coverslips, and not to the surface of the petri-dish and had reached a 20-40% confluency. The coverslips were then removed and their bottom surface glued using a cyanoacrylate adhesive to a standard microscope slide. In order to ensure that the cells remained viable, cells on the top surface were kept moist with PBS (Appendix 1) throughout the assay. The cells were then incubated with 50ul of one of the mouse anti-human antibodies listed in Table 3.1 at a predetermined optimal dilution in PBS (Appendix 1) at 4°C for 45 minutes. As a medium control, two coverslips were incubated with PBS (50ul) alone. After incubation, the slides were washed in a Coplin jar for two minutes with stirring in ice-cold PBS (Appendix 1) to wash off excess antibody that had not bound to the cells. The washing procedure did not affect cell counts on coverslips as 75-80% of the cells remained adherent. Cell viability was also maintained with this treatment. Thereafter cells were incubated with the secondary antibody, an anti-human FITC-conjugated antibody (50ul) at an optimised dilution in

PBS. Of the two coverslips that were incubated with PBS only, one coverslip was incubated with a non-specific antibody (50ul) while the other was incubated with the anti-human FITC-conjugated antibody (50ul, see Section 3.2.3). Cells were incubated at 4°C in the dark. After 45 minutes, the coverslips were washed in a Coplin jar for 2 minutes in ince-cold PBS (Appendix 1) to minimise the amount of non-specific background fluorescence. The coverslip containing adherent cells were covered with another glass coverslip (22x22mm) and the coverslips sealed with clear nail varnish (Cutex, RSA). After these staining perparations, the slides were examined without delay under fluorescence using a fluorescence microscope (Leitz, Germany).

3.3.2 DOSE OPTIMISATION OF PRIMARY AND SECONDARY ANTIBODY CONCENTRATION FOR USE WITH CELL LINES IN IIF ASSAYS

To determine the optimal dosage of both the primary and secondary antibodies required for the most specific fluorescence, a dose dependant IIF was performed using the cell line TONG PHC. Five hundred microlitres of a cell suspension of $1x10^6$ cells/ml in RPMI-1640 supplemented with 10% FCS (complete RPMI) (Section 2.4.2) was added to each well of a tissue culture slide (Labtek, Miles Scientific, USA). Cells were cultured under standard cell

culture conditions. After 18 hours of incubation at 37°C in an humified 95% air-5% CO_2 chamber the medium was gently removed from each well, and the dose dependent assay performed as illustrated in Table 3.2 using the method as described in Section 3.3.1. As washing with PBS or RPMI-1640 did not alter the appearance of fluorescence (Results not shown) all washing was performed with PBS for economy. After staining the cells, the plastic demarcations were removed from the tissue culture slide and the slide sealed with another clear glass slide using clear nail varnish (Cutex, RJA). The slides were viewed under a Leitz fluorescent microscope (Leitz, Germany). The optimal dosage for each monoclonal antibody as well as for the FITC-conjugate as determined in the dose response experiments was adopted for all the IIF experiments performed on HC as well as control cell lines, Raji and K562. The optimal dosage for each antibody is tabulated in Table 3.5.

3.3.3 IIF ASSAY PERFORMED ON RAJI AND K562

3.3.3.1 A Modified IIF Assay Using Viable Cells

Raji and K562 cells were harvested, enumerated and their viability assessed using the trypan blue exclusion test (Section 2.4.3) and the cells brought to a final concentration of 1 x 10^6 cells/ml. After centrifugation at 200 g for 7 minutes at room tempreture, as much fluid as

TABLE 3.2 CHECKERBOARD TITRATIONS FOR OPTIMISATION OF PRIMARY MONOCLONAL ANTIBODIES (MOABs) AND FITC-CONJUGATED SECONDARY ANTIBODY USED IN THE IIF ASSAY

Dilutions of:

MOABs	0	1:5	1:10	1:50	1:100
FITC-conjugate	1:2	W6/32	W6/32	W6/32	W6/32
	1:5	anti-ß2m	anti-ß2m	anti-ß2m	anti-ß2m
	1:10	TAL IB5	TAL IB5	TAL IB5	TAL IB5
	1:50	W6/32	W6/32	W6/32	W6/32

possible was carefully aspirated. The cell pellet was resuspended manually in the remaining supernatant (500 ul). Thereafter, 75ul of the primary antibody at the optimal dose of 1:50 was added to the cell suspension and the suspension gently mixed. After incubation for 45 minutes at 4°C, the tube was filled to approximately 10ml with cold RPMI-1640 (Highveld Biologicals, Jhb. RSA). The cells were recentrifuged at 200 g for 7 min at 4°C and the supernatant removed. The cell pellet was resuspended manually in the remaining supernatant (500ul) and thereafter the cells washed twice at 4°C with cold (4°C) sterile PBS. After the second wash the cell pellet was resuspended manually in the remaining supernatant and the cells incubated in the dark at 4°C with 75ul of the secondary antibody (See Section 3.2.2). After 45 minutes, the cell suspersion was diluted to 10ml with cold PBS and re-centrifuged at 4°C at 200g for 7 minutes. After the removal of the supernatant, the cells were gently resuspended manually and the final volume adjusted to 500ul with PBS. One drop of the cell suspension was placed on a standard glass microscope slide, and the suspension sealed by a glass coverslip (22x22mm) using clear nail varnish (Cutex, RSA). The cells were examined under fluorescence (Leitz, Germany) without delay.

3.3.3.2 A MODIFIED IIF ASSAY USING FIXED CELLS

In previous experiments a number of fixatives were investigated in order to examine the effect of different fixatives on MHC antigen expression. These included ice-cold ethanol, ice-cold acetone and 2% paraformaldehyde at room temperature. Ethanol and paraformaldehyde both caused the disappearance of fluorescence as observed under the fluorescent microscope, however the use of acetone did not interfere with IIF staining. When ice-cold acetone was used as a fixative the following procedure was adopted: 200ul of a 1x 10^6 cells/ml suspension was placed onto a standard microscope slide. After air drying, the slide was immersed in ice-cold acetone for 60 seconds. After further air drying, the cells were exposed to the primary and secondary antibodies as described in Section 3.3.3.1.

3.4 RESULTS

The results obtained by incubating cells from the various HCC lines with the panel of monoclonal antibodies are summarised in Table 3.3. Expression of MHC antigens was scored as "negative" when 20% or less of the hepatoma cells in the field of vision examined spontaneously displayed a specific stain and "positive" when an homogenous stain of the plasma membrane of 80% or more cells in the field of vision could be detected under spontaneous conditions. As shown in Table 3.3 all HCC cell lines investigated

TABLE 3.3 EXPRESSION OF HLA ON HCC LINES[a] INCUBATED WITH MOABs AS DETECTED BY THE IIF ASSAY

MOAB[b]	PLC/ PRF/5	HA22T/ VGH	HA59T/ VGH	TONG	HEP G2	HEP 3B	MAHLAVU
W6/32	+[c]	+	+	+	+	+	+
anti-ß2m	+	+	+	+	+	+	+
TAL IB5	-[d]	+	+	-	-	-	-
VIC Y1	-	+	+	-	-	-	-

a. Refer to Table 2.1 for description of HCC lines.

b. For specificities of MOABs refer to Table 3.1.

c. "+" denotes spontaneous expression.
(ie ≥80% of the cells in the field of vision examined spontaneously fluoresced).

d. "-" denotes negligible spontaneous expression.
(ie ≤20% of the cells in the field of vision examined spontaneously fluoresced).

spontaneously express MHC Class I antigens and its associated invariant chain, ß2-microglobulin. The intensity of staining varied between cell lines and from cell to cell, although in all cell lines used, virtually all cells (approximately 100%) were bound by the anti-HLA Class I and anti-ß2m monoclonal antibodies. As shown in Table 3.3 the Alexander cell line, PLC/PRF/5 and the cell lines Hep G2, Hep 3B, TONG PHC and Mahlavu did not spontaneously exhibit surface MHC Class II antigens using the IIF technique. The cell lines HA22T/VGH and HA59T/VGH exhibited strong staining for both MHC Class II antigens and its associated invariant chain. (See Table 3.3). Representative patterns of IIF staining are shown in Fig 3.1 to Fig 3.5. MHC antigens appeared under fluorescence as fine granules on the cell surface.

Table 3.4 summarises the results obtained using both viable and fixed cells for the IIF assay on the control cell lines Raji and K562. There appeared to be no differences in the MHC surface antigen expression between viable and acetone-fixed Raji and K562 cells as detected by indirect immunofluorescence staining. Using both viable and fixed cells Raji spontaneously expressed both classes of MHC as well as their respective invariant chains. K562 did not spontaneously express MHC antigens (See Table 3.4). This assay therefore confirmed the validity of using the cell

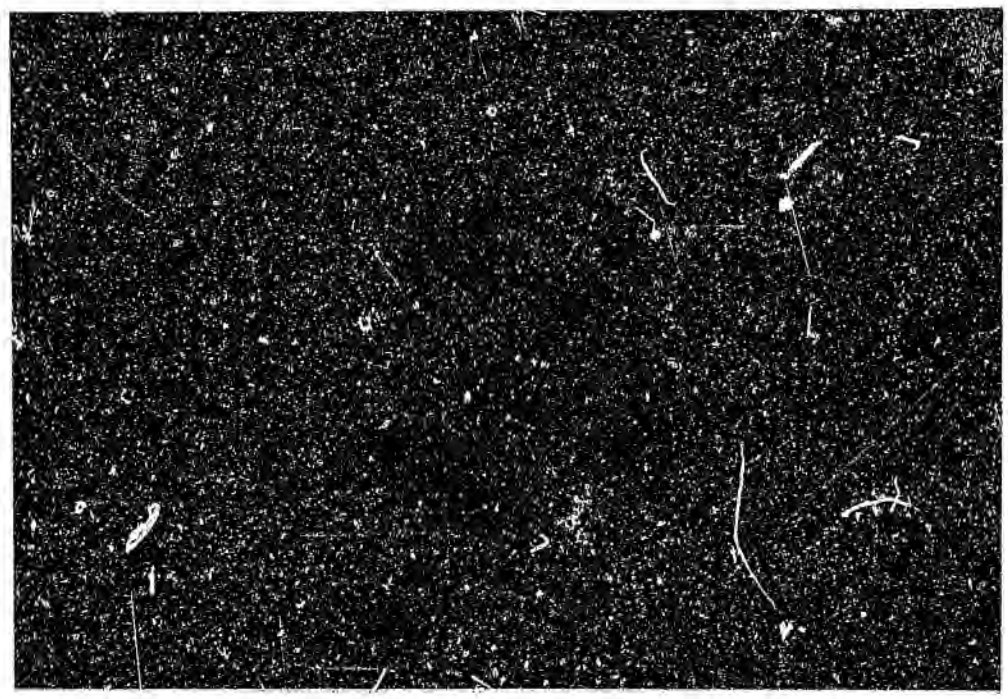


Figure 3.1
Immunofluorescent staining of the human HCC cell line HA22T/VGH demonstrating spontaneous HLA-A,B,C expression as detected with the monoclonal antibody W6/32. The figure demonstrates fluorescence as fine green granules on the plasma membrane. (Magnification 40x)

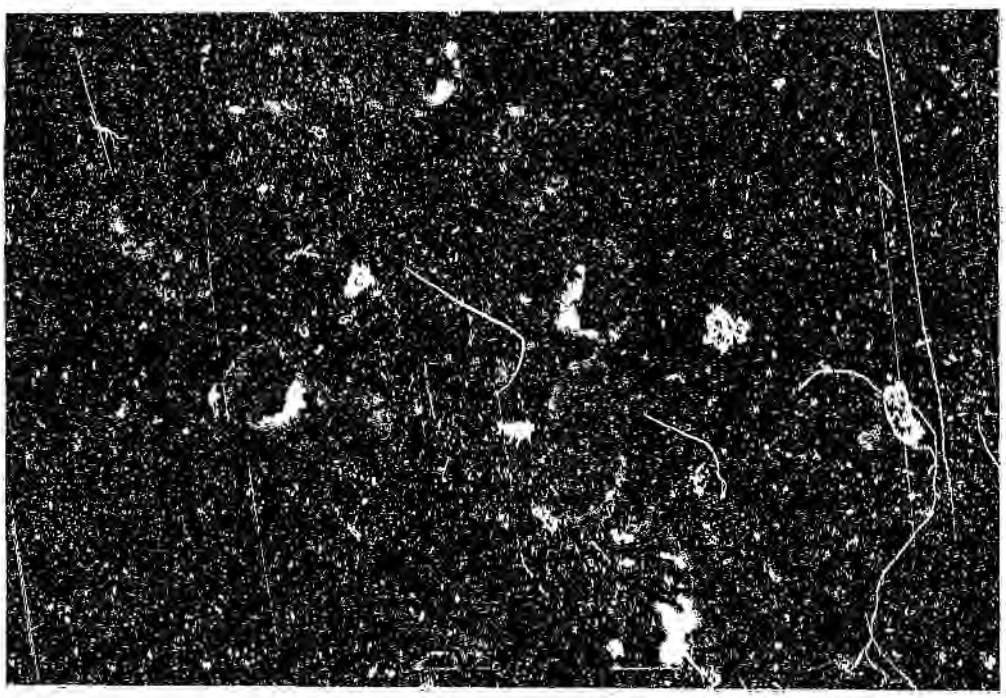


Figure 3.2

Immunofluorescent staining of the human HCC cell line PLC/PRF/5 demonstrating spontaneous HLA-A,B,C expression as detected by the monoclonal antibody W6/32. The figure demonstrates fluorescence as fine granules on the cell surface. (Magnification 40x)

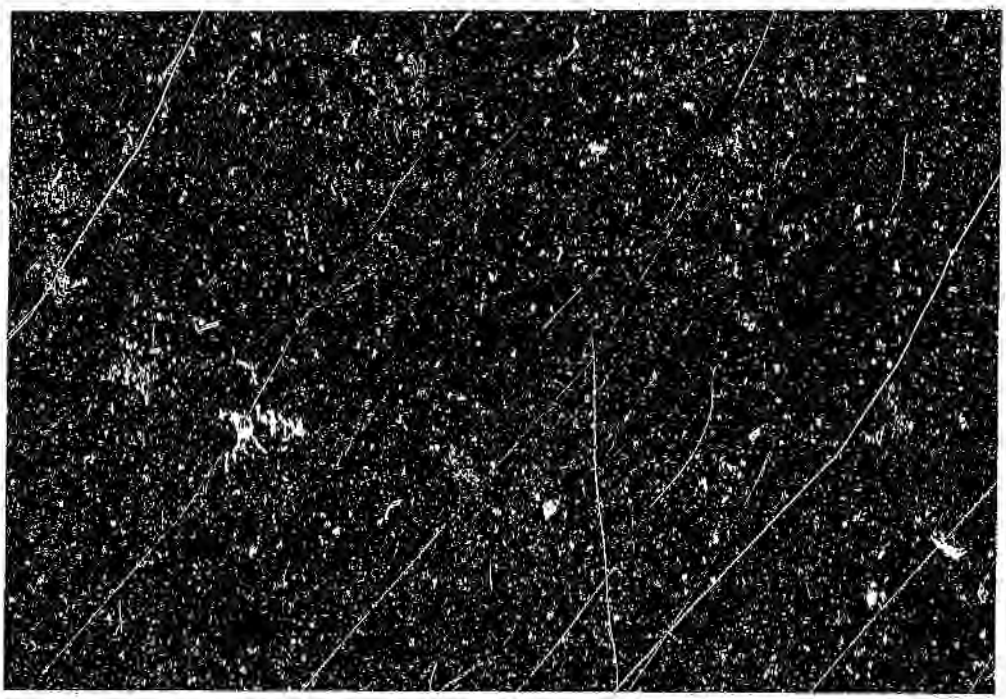


FIGURE 3.3

Immunofluorescence staining to demonstrate the spontaneous presence of beta-2-microglobulin (ß2m) on the human HCC cell line HA22T/VGH. The figure demonstrates fluorescence as fine green granules on the plasma membrane. (Magnification 40x)

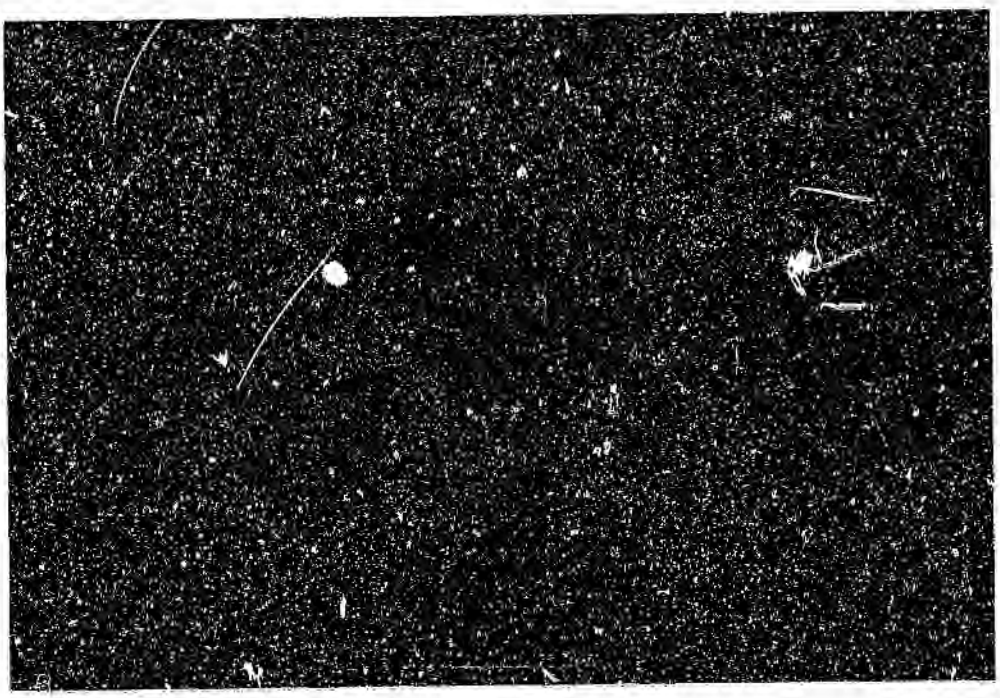


FIGURE 3.4

Immunofluorescence staining to demonstrate spontaneous expression on the human HCC cell line HA22T/VGH. The surface structures were detected by indirect immunofluorescence using the monoclonal antibody TAL IB5 (anti-HLA-DR). (Magnification 40x)

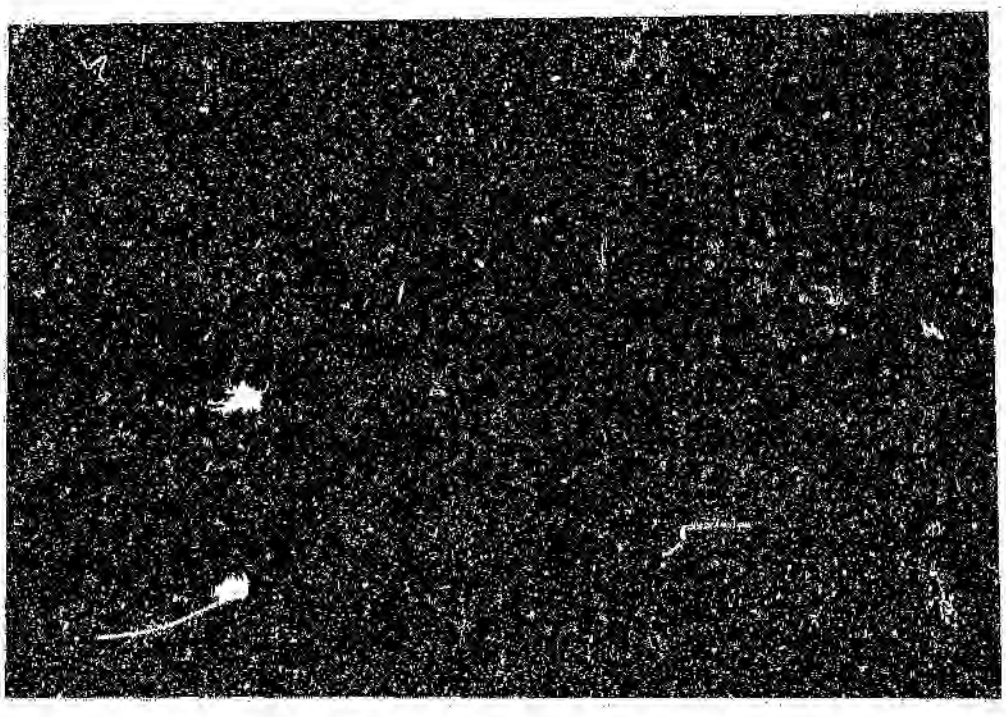


FIGURE 3.5

Indirect immunofluorescence staining to demonstrate spontaneous HLA-D region-associated invariant chain, Ii, expression on the human HCC cell line HA22T/VGH. The figure demonstrates fluorescence as fine green granules on the plasma membrane as detected by the monoclonal antibody VIC Y1. (Magnification 40x).

lines Raji and K562 as positive and negative control cell lines respectively. The HCC cells did not react with normal mouse gamma-globulin or PBS.

The results of the dose response experiments performed on the monoclonal and FITC conjugated antibodies used in this study (Table 3.1) are summarised in Table 3.5. The amount of fluorescence observed for each dilution of MOAB and FITC-conjugated antibody may be summarised as follows: when no MOAB was used for the assay, irrespective of the dosage of FITC-conjugated antibody , no resulting IIF staining was observed. This lack of fluorescence was designated as "0" as seen in Table 3.5. A dilution of the MOABs W6/32 (anti-HLA Class I) and anti-ß2m of 1:5 with a corresponding dilution of 1:2 of the FITC-conjugated antibody resulted in maximal non-specific binding of the MOAB as well as maximal non-specific background fluorescence being observed. The fluorescence observed when these dilutions were used for IIF staining was designated as "3+" as seen in Table 3.5. Similar degrees of fluorescence were also observed for dilutions of MOAB and FITC-conjugated antibody at 1:10 and 1:2, 1:50 and 1:2 and 1:5 and 1:5 respectively. When a specific display of MHC antigens was coupled with a minimal non-specific background fluorescence, the designation of "2+" was assigned. The symbol "+" was assigned to the amount of fluorescence observed when the dilutions of MOAB

TABLE 3.4 SPONTANEOUS EXPRESSION OF HLA CLASS I AND II AND INVARIANT CHAINS ON K562 AND RAJI CELL LINES AS DETERMINED BY THE IIF ASSAY

	Raji		K562	
MOAB[a]	Live	Fixed	Live	Fixed
W6/32	+[b]	+	-[c]	-
anti-ß2m	+	+	-	-
TAL 1B5	+	+	-	-
VIC Y1	+	+	-	-

a. The specificities of each MOABs is described in Table 3.1.

b. "+" implies spontaneous expression (ie ≥ 90% of the cells in the field of vision examined spontaneously fluoresced).

c. "-" implies negligible spontaneous expression (ie ≤10% of the cells in the field of vision examined did not spontaneously fluoresce).

TABLE 3.5 OPTIMAL DILUTIONS OF MOABs BINDING TO HLA-A,B,C, D AND THEIR RESPECTIVE INVARIANT CHAINS, β2m AND Ii, USING THE CELL LINE TONG PHC

MOAB		0	1:5	1:10	1:50	1:100
FITC-conjugate	1:2	-	3+	3+	3+	2+
	1:5	-	3+	3+	+	+
	1:10	no binding with HLA-D region antigens was was observed				
	1:50	-	2+	+	+	+/-

Each MOAB directed against a certain HLA specificity as outlined in Table 3.2 was used at dilutions indicated (0, 1:5, 1:10, 1:50, 1:100). The dilution of the FITC-conjugated antibody used is shown on the vertical axis (1:2, 1:5, 1:10, 1:50). Each FITC-conjugated dilution was used against a different MOAB dilution and FITC-conjugated antibody resulted in maximal specific display of MHC antigens and negligible amounts of non-specific background binding. At these dilutions, the dosage of MOAB and FITC-conjugated antibody was considered optimised. For the monoclonal antibody W6/32

(anti-HLA-A,B,C), a dilution of 1:50 and FITC-conjugated antibody dilution of 1:50 optimal. These dilutions demonstrated the most defined and clear fluorescent pattern. (Fig 3.1 and 3.2). Binding of TAL IB5, an anti-HLA-DR antibody, to any cells of the cell line TONG PHC was not observed at any of the doses used (1:5-1:100). For practical purposes therefore, the dosage of 1:50 for TAL IB5 was employed for further IIF experiments. As seen in Figure 3.5, IIF staining of HA22T/VGH cells using this dilution of TAL IB5 also resulted in a clear and defined fluorescent pattern. The monoclonal antibody, anti-β2m, was optimised at a dilution of 1:10 using a FITC-conjugated antibody dilution of 1:50. An example of the resulting crisp fluorescent pattern is shown in Figure 3.3. The dilution of 1:1000 was employed for VIC Y1 (anti-Ii) according to personal information from W. Knapp. The clear IIF staining resulting from this dilution of the MOAB and a dilution of the FITC-conjugated antibody of 1:50 is shown in Figure 3.5. The assignment of "+/-" was given when the monoclonal antibody was too dilute to maintain its specificity (Table 3.5).

3.5 DISCUSSION

Table 3.3 summarises the spontaneous expression of MHC Class I and II surface antigens as well as their

respective invariant chains, ß2m and Ii. The results of this present study indicated spontaneous expression of MHC Class I and ß2m on all the HCC cell lines investigated. These are consistent with those previously reported (Sung et al 1989, Fukusato et al 1986, Krief et al 198 , Franco et al 1988). All the abovementioned studies employed an indirect immunofluorescence assay as a method of assessing MHC surface expression on the following HCC cell lines: PLC/PRF/5 (Sung et al 1989, Krief et al 1988, Franco et al 1988, Fukusato et al 1986), Hep 3B, Hep G2 (Krief et al 1988, Sung et al 1989, Fukusato et al 1986), Tong PHC (Sung et al 1989), HA22T/VGH (Krief et al 1988, Sung et al 1989) and Mahlavu (Fukusato et al 1986, Sung et al 1989) investigated in this study. The cell lines Hep G2, Hep 3B and TONG PHC did not exhibit spontaneous MHC Class II antigen expression as shown in Table 3.3. Sung et al (1989), Fukusato et al (1986) and Krief et al (1988), using the IIF technique, reported similar results on these three cell lines. The results of the present study demonstrated that the HCC cell line PLC/PRF/5 does not spontaneously express MHC Class II antigens. This finding is in agreement with that of Sung et al (1989), Fukusato et al (1986) and Krief et al (1988) who showed that the Alexander cell line does not express HLA-D region antigens under spontaneous conditions. However, Franco et al (1988) reported that Alexander cells spontaneously express

MHC Class II antigens at low levels as detected by the IIF technique. This discordance in results could be explained by the variations in MHC antigenic profiles of the different generations of the cell line which is expected from cell culture work (Freshney 1987) as well as differences in sensitivity in the methods of detection used.

Krief et al (1988) and Sung et al (1989) reported the constitutive expression of MHC Class II antigens in the less differentiated hepatoma cell line, HA22T/VGH. Similarly, the present study also reports spontaneous expression of MHC Class II in the HA22T/VGH cell line.

MHC Class II and its associated invariant chain, Ii were both spontaneously expressed in the cell line HA59T/VGH as assessed by the IIF technique in this study. The status of MHC antigen expression in this HCC cell line was not available from the literature.

Cells that were labelled with a non-specific antibody directed against the heavy chains of all the classes of the immunoglobulins in order to assess the specificity of the IIF technique did not exhibit any fluorescence. This indicates that the fluorescence observed on HCC cells was indeed because of the immunolabelling of specific MHC

antigens on the plasma membrane of these cells. In order to assess the sensitivity of the assay, cells used for this assessment were labelled with the secondary but not primary antibody. That no fluorescence was observed on these cells implied firstly, the monoclonal antibodies were conjugating with specific MHC antigens on the cell surface and not to random cell surface receptors such as glycoproteins and secondly, the FITC-conjugated immunoglobulin $F(ab^1)_2$ fragment was labelling the monoclonal antibodies and not undefined antigens on the cell surface.

In Table 3.4 the results of MHC surface expression for the cell lines Raji and K562 are summarised. The cell line Raji was employed as a positive control cell line because it spontaneously expresses HLA-A,B,C,D antigens as well as the invariant chains, β2m and Ii. Raji has been consistently reported to express MHC Class I and II antigens spontaneously (Nilsson et al 1974). K562, an erythromyeloid-derived cell line, did not spontaneously express MHC antigens as seen in Table 3.4. This cell line has been typed as expressing no significant amounts of MHC surface antigens under resting or unstimulated conditions (Lozzio & Lozzio 1975). For the above reason, K562 was used as a negative control in this study. Thus the IIF assay confirmed the validity of the assignment of these

two cell lines as controls for the present study. The results of the optimisation of the monoclonal antibodies and FITC-conjugated antibody are summarised in Table 3.5. Optimal fluorescence was considered when maximal specific binding to an HLA specificity was coupled with negligible non-specific background fluorescence. The optimisation of antibodies used in indirect immunofluorescent staining is necessary because the specific activities of each monoclonal antibody is different, resulting in maximal binding of the antibody occurring at different dilutions. The monoclonal antibody W6/32 (anti-HLA-A,B,C) exhibited maximal antigen-antibody binding at a dilution of 1:50. A dilution of 1:100 was too high to be of any experimental use. Similar results were observed for the monoclonal antibody, anti-β2m but for this MOAB, a lower dilution was used experimentally (1:10) as fluorescence at a higher dilution seemed to fade more quickly.

The results of the present study correlated with those of Boucheix et al (1985) in that approximately 10% of the cells used in experiments were lost after 2 washings and 2 incubations. Viability decreases by less than 10% in the course of the experiment (results not shown).

Staining of cell surface antigens with corresponding labelled antibody frequently fails when an inappropriate

fixative is used. (Jessen 1983). This is because of changes in the distribution and shape of the antigen as well as cross linking of membrane proteins (Jessen 1983). Since MHC antigens are surface antigens and can therefore be stained directly with labelled antibody (Jessen 1983), this study compared MHC antigen surface expression on viable and fixed cells. The effect of fixation was investigated by the use of different fixatives namely ethanol, paraformaldehyde and acetone and the optimal fixative assessed. The results of MHC surface expression on viable and fixed cells are summarised in Table 3.4 using ice-cold acetone as the fixative. As seen in Table 3.4 there was no differences in the expression of HLA Class I and II as well as their respective invariant chains using both viable and fixed cells when acetone was used as a fixative. No fluorescence was observed using indirect immunofluorescence staining when paraformaldehyde and ethanol were used as fixatives.

It is worth discussing why the fixatives ethanol and paraformaldehyde were not suitable for the fixation of control cells in the immunofluorescence assay. Paraformaldehyde is a cross-linking fixative which reacts in a complex manner with cell proteins (Jessen 1983). This results in the formation of links between adjacent protein chains, thus effectively permeabilising cell

membrane to antibody molecules (Jessen 1983). The degree of permeability depends on the concentration of fixative used with a higher concentration being less successful in achieving permeability than a lower one (Jessen 1983). The permeabilisation of the cell membrane before addition of antigens is not optimal when detecting cell surface antigens since it mostly leads to weaker immunostaining and can cause higher background fluorescence through non-specific antibody interactions with extracellular components (Jessen 1983). Thus when a 2% paraformaldehyde aqueous solution was used as fixative for control cells K562 and Raji, it could have been at too high a concentration and thus destroyed the cell structures responsible for antibody reactions. Another possible cause of an absence of fluorescence being observed when 2% paraformaldehyde was used, could be that the high background fluorescence (for reasons described above) made observation of MHC antigen staining difficult. Similar reasons could have been responsible for 96% EtOH not being an effective fixative in the system used in this study. Ice-cold acetone is a classic fixative for immuno-cytochemical procedures and in this study, proved to give the best results as a fixative in the indirect immunofluorescence assay of Raji and K562 cells.

During the immunofluorescence assay, cells were incubated

at 4°C for several important reasons. Firstly, Pernis and Tse (1985) have detailed evidence that the recycling of MHC Class I in activated T lymphocytes occurs at 37°C. Pernis and Tse (1985) have demonstrated that MHC molecules are continuously internalised or endocytosed and that cells that are carrying MOAB, or are FITC-labelled have these labels recycled with them. They have also demonstrated that the time necessary for the internalisation of 50% of the surface MHC Class I antigens is approximately 60 minutes, while the resurfacing of the recycled antigens takes approximately 20 minutes (Pernis & Tse 1985). However, this recycling process is slowed down considerably at 4°C (Pernis & Tse 1985). Incubation at 4°C also slows down cell metabolism, preventing such phenomenon as capping which is the redistribution of cell surface molecules (such as HLA) when cross-linked or exposed to antibodies (Klein 1986). The mechanism of capping has yet to be elucidated but it is an energy requiring process (Klein 1986). Capping is blocked by inhibitors of metabolism such as sodium azide, thus most, including the Moabs used in this study, are co-diluted with sodium azide. Antibody-induced redistribution of MHC molecules can result in the effective disappearance of these antigens (due to internalisation of antigen-antibody complexes) from the cell surface thus giving rise to false negative results. The use of sodium azide together with performing the IIF assay at 4°C ensured that this phenomenon did not occur.

CHAPTER 4

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

4.1. Introduction

The enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA) is a sensitive assay which is increasingly being used to detect specific antigens (such as malaria) occurring in low concentrations (Voller et al 1976). EIA and FLISA's are probably the most widely used of all immunological assays for detecting antibodies to various infectious agents since large numbers of tests can be performed in a relatively short time with these tests (Roitt et al 1989).

The rationale behind the enzyme immunoassay is that an antigen or antibody can be linked to an enzyme whilst retaining both immunological and enzymatic activity in the resulting conjugate (Roitt et al 1989). In 1964, Nakane and Pierce demonstrated chemical linkage between enzyme and antibody, indicating the effective use of an indirect immunoenzyme assay. In 1970, Sternberger et al reported that the immunological linkage of a peroxidase-bound enzyme with antibody was biochemically possible. Sternberger et al (1970) developed a quantitative assay of the antibody against the spirochete Treponema pallidum. Later

workers in this field concentrated on the visual detection and localisation rather than the immunological aspects of antigens (Engvall & Perlman 1972, Engvall et al 1971, van Weeman & Schuurs 1971). Engvall and workers (Engvall & Perlman 1972, Engvall et al 1971) and van Weeman & Schuurs (1971) developed techniques for quantitating specific antibodies using an alkaline-phosphatase labelled and horseradish peroxidase-labelled enzyme respectively. In these assays, the soluble antibodies were linked to an insoluble solid phase containing antigen in such a way that the reactivity of the immunological component of the antigen-antibody interaction was retained. The reagents used in these assays are highly stable, have a long shelf life and present no health hazards (Watt et al 1988). Three general types of ELISA have been developed: a) the direct method, in which antibody is detected and measured, b) the double antibody sandwich and c) the competitive method. For the latter two assays antigen is immobilised on a solid phase, and bound to an enzyme-labelled antibody which is then detected colorimetrically. The sandwich method of performing the ELISA was adopted in this study, since the protocols of this technique were already established in the laboratory.

4.1.2 GAMMA-INTERFERON

The existance of a substance that conferred resistance to viral infections was demonstrated as early as 1957 by Isaacs & Lindenmann. Since then, the family of interferons have been shown to be potent biomodulators involved in antiviral activities, cellular differentiation and proliferation (Branca 1988, Revel & Chebath 1986, Rosa & Fellous 1984). Interferons are highly heterogenous in their molecular structure (Duc-Goiran et al 1986), with three major classes of interferons having been described. These are: leukocyte or alpha interferon, fibroblast or beta interferon and immune or gamma interferon (Pestka et al 1987) with all classes being present in all mammalian species (Pestka et al 1987).

Human IFN-alpha, -ß and -gamma have been shown to have the ability to induce a common set of proteins and activation of genes including MHC Class I genes (Marley et al 1989, Anderson & Berkowitz 1985, Ottesen et al 1990, Driggers et al 1990) and ß2-microglobulin (Marley et al 1989, Anderson & Berkowitz 1985, Ottesen et al 1990, Driggers et al 1990) as well as those encoding for 2',5'-oligoadenalyte synthetase (Eades et al 1990). However IFN-gamma, synthesised by activated lymphocytes acts through a distinctly different receptor (Weil et al 1983, Revel & Chebath 1980) and differs in cell origin, inducing agents,

physical and biological properties, and amino acid sequence (Gray et al 1982) to IFN alpha and -ß.

Interferon-gamma has been shown to be capable of increasing the production of antibodies by B lymphocytes both in vivo and in vitro (Nakamura et al 1984). In addition, it has been demonstrated that IFN-gamma is involved in activating genes encoding for MHC Class II proteins and their associated invariant chain (Ii) (Pessara et al 1988, Ucer et al 1985, Sugimoto et al 1989, Basham & Merigan 1983, Koch et al 1984), suggesting specialised role for IFN-gamma in immune regulation (Basham & Merigan 1983). It has been previously reported (Krief et al 1988, Yoshioka et al 1988) that incubation of hepatoma cells with IFN-gamma enhanced the expression of HLA Class I and II expression on these cells. This present study, therefore, undertook to determine whether recombinant-IFN-gamma and -alpha exerted an effect on MHC expression on the HCC cell lines investigated in this study.

4.1.3 SODIUM BUTYRATE

Sodium butyrate is a physiologically occurring fatty acid that has been shown to have a wide variety of effects on mammalian cells in culture (Prasad & Sinha 1976, Candido et al 1978). Initially, it was shown by Leder & Leder (1975)

that butyric acid was capable of inducing globin gene expression in Friend erythroleukaemic cells. Evidence accumulated over the past few years suggests that butyrate may also be an important pharmacologic agent for studying the mechanisms of gene expression (de Haan et al 1986, Prasad & Sinah 1976). It has been shown that butyrate causes increased acetylation of histones H3 and H4 as well as alterations in chromatin structure and activity (Kitzis et al 1980). de Haan et al (1986) and Riggs et al (1977) reported that sodium butyrate inhibits DNA synthesis, and induces hypermethylation of DNA in normal and transformed embryonic lung fibroblast cell lines thus affecting gene expression. Since butyrate effect different genes and different cell types in a tissue- specific manner, sodium butyrate may be capable in both inducing and/or repressing expression of genes (de Haan et al 1986).

As a differentiation modifier (Marks & Rifkind 1984), sodium butyrate has the most consistent effect of inducing the differentiation of transformed cells in culture into more mature or "normal" cells (Engelmann et al 1987). Because butyrate is a nontoxic and antiproliferative compound, it has been suggested that it may be valuable in the chemotherapeutic treatment of solid tumours as well (Engelmann et al 1987, Kaneko et al 1990). Kaneko and co-workers also demonstrated that Chang but not PLC/PRF/5

and Hu-7 human hepatoma cell lines were transformed into fibroblast-like cells with a markedly elongated cytoplasm when treated with novobiocin, a topoisomerase inhibitor, and butyrate (Kaneko et al 1988,1990) . Nakagawa et al (1985) demonstrated that butyrate induced a significant, dose-related inhibition of PLC/PRF/5 cell growth. Morphological changes were revealed using Giemsa-staining of cells treated with 1mM sodium butyrate (Nakagawa et al 1985). These workers also demonstrated that butyrate caused a significant decrease in the total accumulated extracellular alpha-fetoprotein (AFP) level in PLC/PRF/5 (Nakagawa et al 1985). Cook et al (1985) similiarly reported that sodium butyrate decreases AFP mRNA levels in rat hepatoma cells in vitro.

The mechanisms of action of sodium butyrate on various cell lines are currently unknown (Prasad & Sinha 1976). However, Sutherland et al (1985) while investigating the pattern of differentiation in the K562 cell line initiated by sodium butyrate, observed that after incubation with sodium butyrate, HLA Class I antigens were detectable on the cell membrane. It appears that the mechanism by which sodium butyrate and gamma interferon induce HLA expression is under genetic control. Sutherland et al (1985) detected an increment in the level of HLA-A,B,C mRNA in K562 cells that had been pretreated with sodium butyrate for 3 days

and thereafter incubated with IFN-gamma for 48 hours. Moreover it appeared that sodium butyrate and gamma interferon synergistically induced HLA Class I expression (Sutherland et al 1985).

Since human leukaemic cell lines (such as K562) can be seen as models of certain stages of normal differentiation (Bakhski et al 1983), it is possible that the expression of HLA antigens might be related to some process of differentiation (Sutherland et al 1985). For this reason the present study undertook to investigate the effect of sodium butyrate on the status of HLA expression on human hepatoma cell lines.

4.1.4 CLOFAZIMINE

Clofazimine, also known as lamprene or B663 (See Fig 4.1) is an effective anti-leprosy agent (Barry et al 1957, Vischer 1969, Yawalker & Vischer 1979). Apart from its direct anti-microbial properties, clofazimine has been shown to stimulate phagocytic and microbicidal activities (Brandt & Svensson 1973) of human and murine neutrophils and macrophages by potentiating the activity of lysosomal enzymes (Cline 1970, Sarracent & Finlay 1982). Clofazimine also possesses anti-inflammatory and immunosuppressive properties (Gatner et al 1982, Anderson 1985, Anderson et al 1976).

Clofazimine : Chemical structure

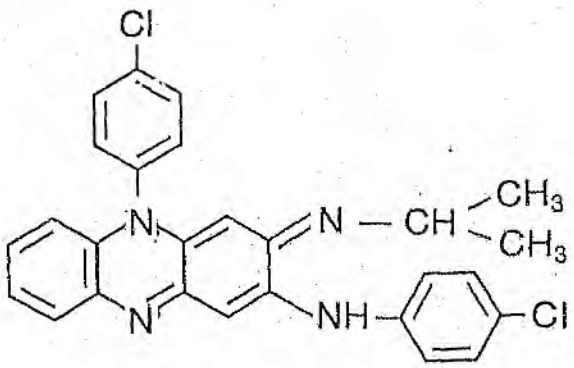


Figure 4.1: Redrawn from Vischer (1969)

It has been found that in mycobacterial diseases such as **mycobacterium tuberculosis**, the capacity of phagocytes for intracellular killing is inhibited (Wadee et al 1987) It has been reported that clofazimine can reverse the inhibitory effects of **mycobacterium tuberculosis** on phagocytic intracellular killing mechanisms (Wadee et al 1988). Similar results were reported by Cline (1970) who demonstrated potentiation of the killing of **listeria monocytogenes** by macrophages. In phagocytes this potentiation causes lysosome production, release of oxygen metabolites such as hydrogen peroxide as well as increased activity of the hexose-monophosphate shunt (HMPS) (Wadee et al 1988). Clofazimine has been demonstrated to prime macrophages for expressing HLA-DR antigens (Wadee AA personal communication). Because of such priming by clofazimine, the present study undertook to examine the effect of clofazimine on the HLA expression of various HCC cell lines.

4.2. MATERIALS

4.2.1. Primary antibodies

Monoclonal antibiodies used in this study are listed in Table 3.1 (page 82).

4.2.2 Secondary antibodies

The peroxidase-labelled antibodies used in this study were

a) horseradish peroxidase-linked sheep anti-mouse Ig whole antibody (Amersham International, Amersham UK)

b) peroxidase-conjugated goat anti-mouse (Cappel Labs, Cochranville, USA.)

4.2.3 LYMPHOKINES AND CHEMICALS

All lymphokines and chemicals were made up as a stock solution maintained in RPMI-1640 (Highveld Biologicals, Johannesburg, SA), filter sterilised through a 0,22 um filter (Millipore, Bedford, MA, USA) and the resulting solution stored at 4°C until used.

a) Chemicals :-

Clofazimine (A gift from R Anderson, University of Pretoria) provided as a stock solution of 200ug/ml in 1% DMSO

Sodium butyrate (Merck Chemicals, Munich, Germany) Stock solution of 50 mM in H_2O

b) Lymphokines:-

Alpha Interferon, IFN-alpha (Roche, Basel, Switzerland) having a specific activity 3 x 10^6 U/ml A stock solution of 3000 IU/ml in RPMI-1640 was used.

Gamma interferon, IFN-gamma (Amersham International, Amersham, UK) a human recombinant secretory form, having a specific activity of 1-5 x 10^7 U/mg. A stock solution of 10000 IU/ml in RPMI-1640 was used.

4.3 METHODS

4.3.1 Determination of MHC antigen expression on HCC cell lines by the ELISA

Confluent monolayer HCC cells were trypsinized, counted on a hemacytometer and their viability assessed by the trypan blue exclusion test (Chap 2.5). Only cells having a 75% confluency and viability were used for this assay. The ELISA was performed in flat-bottomed 96-well culture plates (Cel-Cult Sterilin, Hounslow, UK). All procedures were performed at room temperature unless otherwise stated. The incubation times used in these experiments were previously optimised in this laboratory (A. A. Wadee-personal communication). The plates were washed four times with PBS-0,5% Tween (Appendix 1) between each step. Ten thousand cells of the various HCC cell lines (Table 2.1) in complete DMEM (Section 2.4) were added to each well and the plates were incubated in 95% air-5% CO_2 in a 37°C incubator. After 24 hours the cells were seen to have adhered to the bottom of the well and they had reached 85% confluency. Thereafter the cells were fixed for 30 minutes at room temperature with 100 ul 0,5% paraformaldehyde such that the final concentration of paraformaldehyde was 0,25%. After three washings with PBS-Tween, unbound sites on the surface of each well was blocked with 0,5% Bovine Serum Albumin (BSA) in carbonate buffer at a pH of 9.6 (Appendix 1) and the plates were incubated at room

temperature for 60 minutes. The plates were washed four times in PBS-Tween and 100ul of an appropriately diluted primary antibody in PBS-Tween then added to each well. The antibodies, W6/32 (anti-HLA -A,B,C), TAL IBS (anti-HLA-DR), anti-ß2m and VIC Y1 (anti-Ii) were employed as the primary antibody in the ELISA. After incubation at room temperature for a further 2 hours followed by four washings with PBS-Tween, the cells were incubated with 100 ul of the enzyme-labelled antibody (Section 4.2.2) appropriately diluted in PBS-Tween. Since Tween is an anionic detergent, it blocks electrostatic interactions thus avoiding non-specific bindings between antibody and cell membrane proteins. All dilutions of primary and secondary antibodies were therefore made in PBS-Tween. Thereafter, cells were incubated in the dark with 100ul of a peroxidase substrate, O-phenylenediamine (OPD), in the presence of a 0,03% H_2O_2 solution substrate buffer (Appendix 2). The colorimetric reaction, measuring the extent of antibody binding, was stopped after 15 minutes with 50ul 2,5M H_2SO_4. The optical density (OD) of each well was read at a wavelength of 492 nm on an ELISA Multiskan Titertek Spectrophotometer (Flow Laboratories, Irvine, Ayrshire, Scotland)

4.3.1.1 DETERMINATION OF NON-SPECIFIC BACKGROUND DENSITY

The control blanks used in the assay were as follows: to the wells of the first column in every plate, only cells

and the peroxidase substrate (OPD) were added. To the wells of the second column in every plate, cells, enzyme-labelled antibody and OPD were added. In order to obtain a value for non-specific binding the mean of the values of the first column were subtracted from the mean of the second column. This value was now used as a cell blank. The blank was subtracted from all OD mean values before these results were subjected to statistical analyses. It was observed that the amount of non-specific background density appeared to increase as the shelf life of the peroxidase-labelled conjugate lengthened. For this reason, two different peroxidase-labelled conjugates were used in this study.

4.3.1.2 DETERMINATION OF OPTIMAL ANTIBODY CONCENTRATIONS REQUIRED TO DETECT SURFACE MHC ANTIGENS

In order to standardise and determine the optimal conditions for the ELISA, particular reference was given to two essential variables, namely the concentration of the primary (monoclonal antibody) and the secondary antibody (enzyme-labelled conjugate). Checkerboard ELISA's were performed on the following HCC cell lines: PLC/PRF/5, HepG2, Hep3B, HA22T/VGH, HA59T/VGH, TONG PHC and Mahlavu in order to establish the optimal dilution of primary and secondary antibodies. In these experiments the monoclonal antibodies W6/32, anti-B2m and TAL IB5 were diluted to 1:10, 1:50, 1:100 and 1:200 dilutions in PBS-Tween

(Appendix 1). The monoclonal antibody VIC Y1 was diluted to 1:500, 1:1000, 1:1500 and 1:2000 dilutions in PBS-Tween (Appendix 1). Plates were coated with 10 000 cells in 100ul of complete DMEM and incubated for 24 hours at 37°C in a humidified incubator (95% air-5% CO_2). Cells were fixed with a final concentration of 0,25% paraformaldehyde and unbound sites blocked with 0,5% BSA (Appendix 1). Following four washes with PBS-Tween (Appendix 1), the various dilutions of the monoclonal antibodies (Table 3.1) were added to quadruple wells. After plates had been incubated at room temperature for 2 hours they were washed four times with PBS-Tween. Enzyme-labelled conjugate at various dilutions was added to the wells, such that each of the dilutions of the MOAB was incubated with each dilution of the secondary antibody. The plates were then incubated for 60 minutes at room temperature. After a final four washes, 100 ul of the peroxidase substrate buffer (Appendix 2) were added and the reaction terminated after 15 minutes with 50ul of 2,5M H_2SO4 (Appendix 1). Optical densities were read at 492nm on an ELISA multiskan Titertek spectrophotometer (Flow Laboratories, Irvine, Ayrshire, Scotland).

4.3.2. DETECTION OF SURFACE ANTIGENS ON FIXED CELLS OF THE CELL LINES K562 AND RAJI BY ELISA

Raji and K562 were used as positive and negative controls

respectively in this study. Raji spontaneously expresses MHC Class I and II antigens and K562 expresses only negligible amounts of these antigens. After viability assessment (only cells in suspension with a 85% or higher cell viability were used) and enumeration, cells were plated at a final concentration of 1×10^4 cells/well in 100 ul of complete RPMI in 96-well flat-bottomed cell culture plates (Cel-Cult, Sterilin Ltd, Hounslow, UK). After plating, the plates were immediately centrifuged at 200g in a modified plate centrifuge for 5 minutes. The supernatant was gently aspirated and the cells fixed by the addition of 100 ul of 5% formalin at room temperature for 60 minutes.

Five percent formalin was used as a fixative of Raji and K562 cells since glutaraldehyde and paraformaldehyde (even at final concentrations of 5%) were not as effective fixatives. The cells did not remain adherent to the bottom of the wells when these fixatives were used. Thereafter, plates were centrifuged again at 200 g for 5 minutes. After gentle washing of the plates, unbound sites were blocked with 0,5% BSA in a carbonate buffer (Appendix 1) at room temperature for 60 minutes. Subsequent steps in performing the ELISA were as described in section 4.3.1.

4.3.3 THE EFFECTS OF r-IFN-ALPHA, r-IFN-GAMMA, CLOFAZIMINE AND SODIUM BUTYRATE ON HCC CELL LINE EXPRESSION OF MHC ANTIGENS

Confluent HCC monolayer cells were trypsinized, counted on a hemacytometer and their viability assessd by the trypan blue exclusion tests as outlined in Section 2.5. Of a single cell suspension, 100ul of a stock solution of $4x10^5$ cells/ml made up in Dulbecco's MEM supplemented with 10% FCS (Both from Highveld Biologicals, Johannesburg, SA, Section 2.4) was added to each well. The cell density of 40000 cells/well resulted in a plating confluency of 50 percent. When cell densities higher than this were used, the majority of cells lifted from the surface of the wells after 72 hours. The plates were incubated in a humidified incubator of 95% air-5% CO_2 at 37°C. After 24 hours, various dilutions of the lymphokines r-IFN-gamma and -alpha and the chemicals clofazimine and sodium butyrate (Section 4.2.3) were added to the wells using a checkerboard experimental design and the plates incubated at 37°C in an humidified (95% air-5% CO_2) chamber. In order to determine the optimal time required for maximal stimulation of cells to be achieved plates were removed from the incubator at 4, 18, 24, 48, 72, 96 hours and the cells fixed with a final concentration of 0,25% paraformaldehyde. The plates were incubated at room temperature for 30 minutes and thereafter at 4°C until required (maximal time being 96 hours). The ELISA was performed on all

plates simultaneously. After fixing, the plates were incubated with 0,5% BSA in a carbonate based buffer at room temperature for 60 minutes. Four washings with PBS-Tween were followed by incubation with the panel of monoclonal antibodies directed against MHC Class I and II antigens and their associated invariant chains using predetermined optimal dosages (Section 4.3.1.2). Thereafter the plates were washed four times with PBS-Tween and the cells incubated with the peroxidase-labelled antibody at room temperature for 60 minutes. Subsequent steps in performing the ELISA were described in Section 4.3.1. The optical densities of each well was measured at 492 nm on an ELISA Titertek Multiskan spectrophotometer (Flow Laboratories). Values obtained for wells containing cells that had not been stimulated were subtracted from wells containing the lymphokine or chemical stimulated cells. This reading was used to indicate any change of MHC expression that these agents had caused on the cells.

4.3.3.1 ASSESSMENT OF THE OPTIMAL LYMPHOKINE AND CHEMICAL CONCENTRATIONS REQUIRED TO STIMULATE MHC ANTIGEN EXPRESSION

To determine the optimal concentrations required for the expression of MHC antigens, various concentrations of r-IFN-gamma, sodium butyrate and clofazimine were added to wells containing 1 x 10^4 cells. Recombinant-IFN-gamma was used at a concentration of 50, 100 and 200 IU/ml,

sodium butyrate at dilutions of 1, 2 and 5 mM and clofazimine at 0, 5, 1 and 2 ug/ml. Recombinant-IFN-alpha was used at a concentration of 200 IU/ml. Experiments were performed on the HCC cell lines HA22T/VGH, HA59T/VGH and Tong PHC. The monoclonal antibodies W6/32 (anti-HLA-A,B,C), TAL IB5 (anti-HLA-DR) anti-B2m, VIC Y1 (anti-Ii) were used for the detection of MHC antigens (All monoclonal antibodies were diluted in PBS-Tween). All results reported represented a mean of at least three different experiments.

4.4 RESULTS

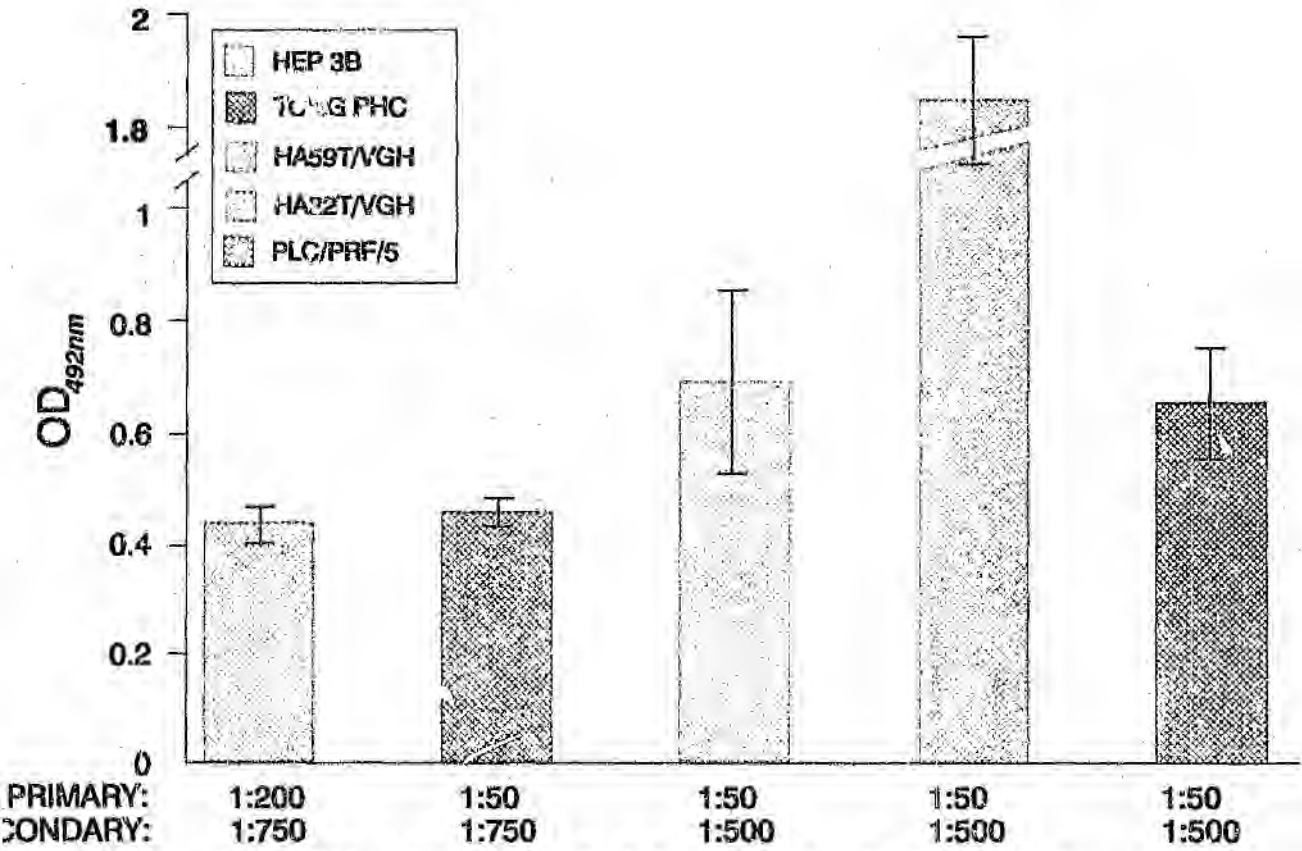
4.4.1 Determination of Optimal Antibody Concentration for use in ELISA

The optimal dose response of the monoclonal antibodies W6/32 and the enzyme-labelled or secondary antibody observed for the HCC cell lines PLC/PRF/5, TONG PHC, HA59T/VGH, HA22T/VGH and Hep 3B are shown in Figure 4.2. On each of these cell lines a definite spontaneous expression of MHC Class I antigens as detected by the MOAB W6/32 was observed. (Results of Mahlavu and Hep G2 not shown). As shown in Fig. 4.2, the dilution of the primary and secondary antibody at which maximal detection of MHC Class I antigens occurred on the cell lines PLC/PRF/5, HA59T/VGH and HA22T/VGH was at dilutions of 1:50 and 1:500 respectively. The optimal dilutions of the primary and

secondary antibody on the cell line, Hep 3B, occurred at dilutions of 1:200 and 1:750 respectively, while on the cell line TONG PHC, optimisation of primary and secondary antibodies occurred at dilutions of 1:50 and 1:750 respectively (Fig 4.2).

In order to determine the optimal concentrations of both the primary and secondary antibody that would be required for maximal resolution of MHC Class I antigens by the ELISA, dose response experiments were performed using varying dilutions of the monoclonal and enzyme-labelled antibody. Figures 4.3 to 4.7 demonstrate the dose response curves obtained for the HCC cell lines Hep 3B, TONG PHC, HA59T/VGH, HA22T/VGH and PLC/PRF/5 respectively. These graphs show the differing optical density values obtained for every dilution of the primary and secondary antibody used in this study. Each cell line exhibited a different dose response curve.

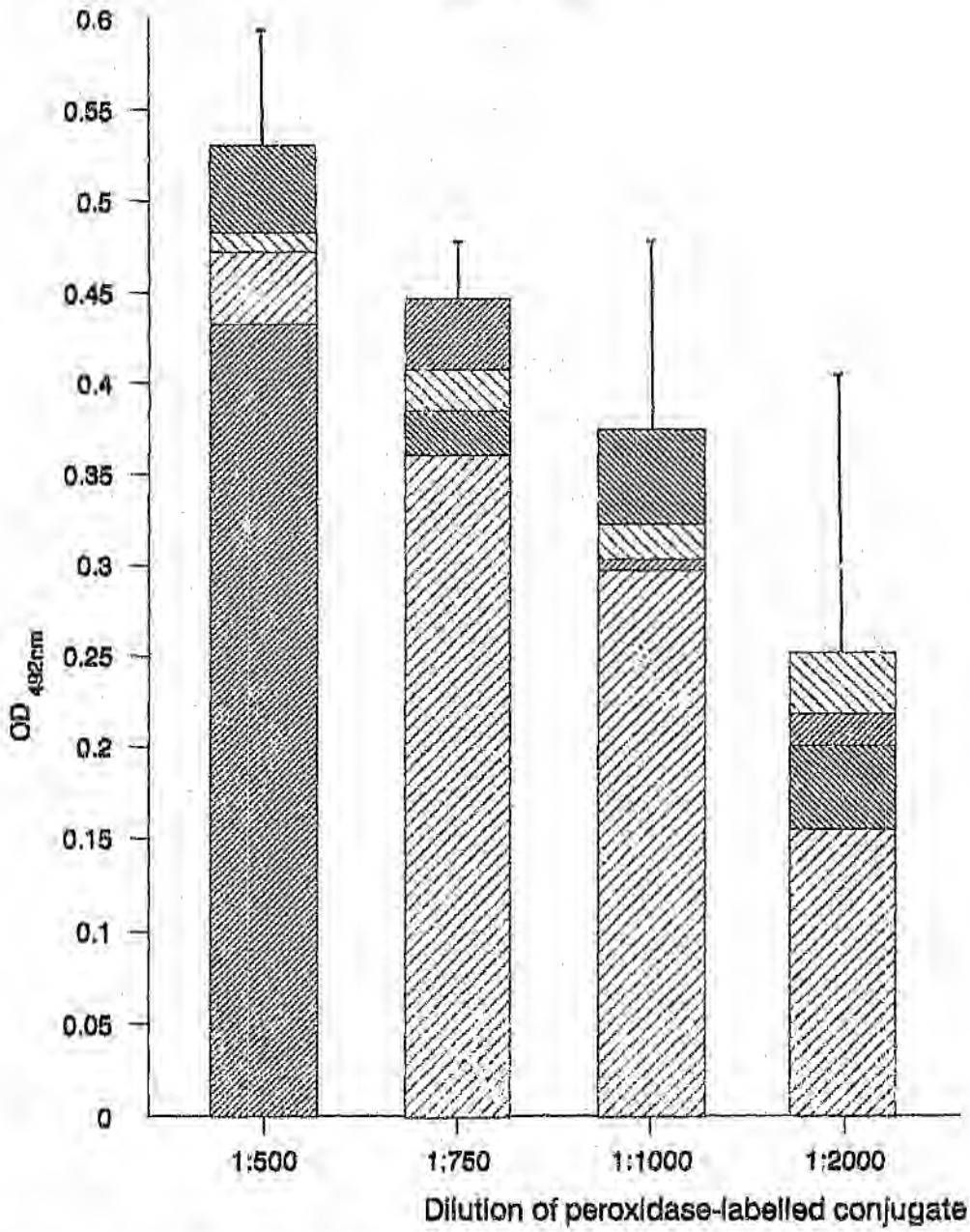
On the cell line, Hep 3B, the optimal dilution of the primary antibody was observed to be 1:200 while optimisation of the secondary antibody dilution occurred at 1:750 (Figure 4.3). A dilution was regarded as optimal when the optical density measured for that particular dose of the primary or secondary antibody was raised with a standard deviation of less than 0,15 when compared with



OPTIMAL DOSE RESPONSE OF

1)PRIMARY ANTIBODY: ANTI-HLA-A,B,C (W6/32)
2)SECONDARY ANTIBODY: PEROXIDASE-LABELLED CONJUGATE

Figure 4.3

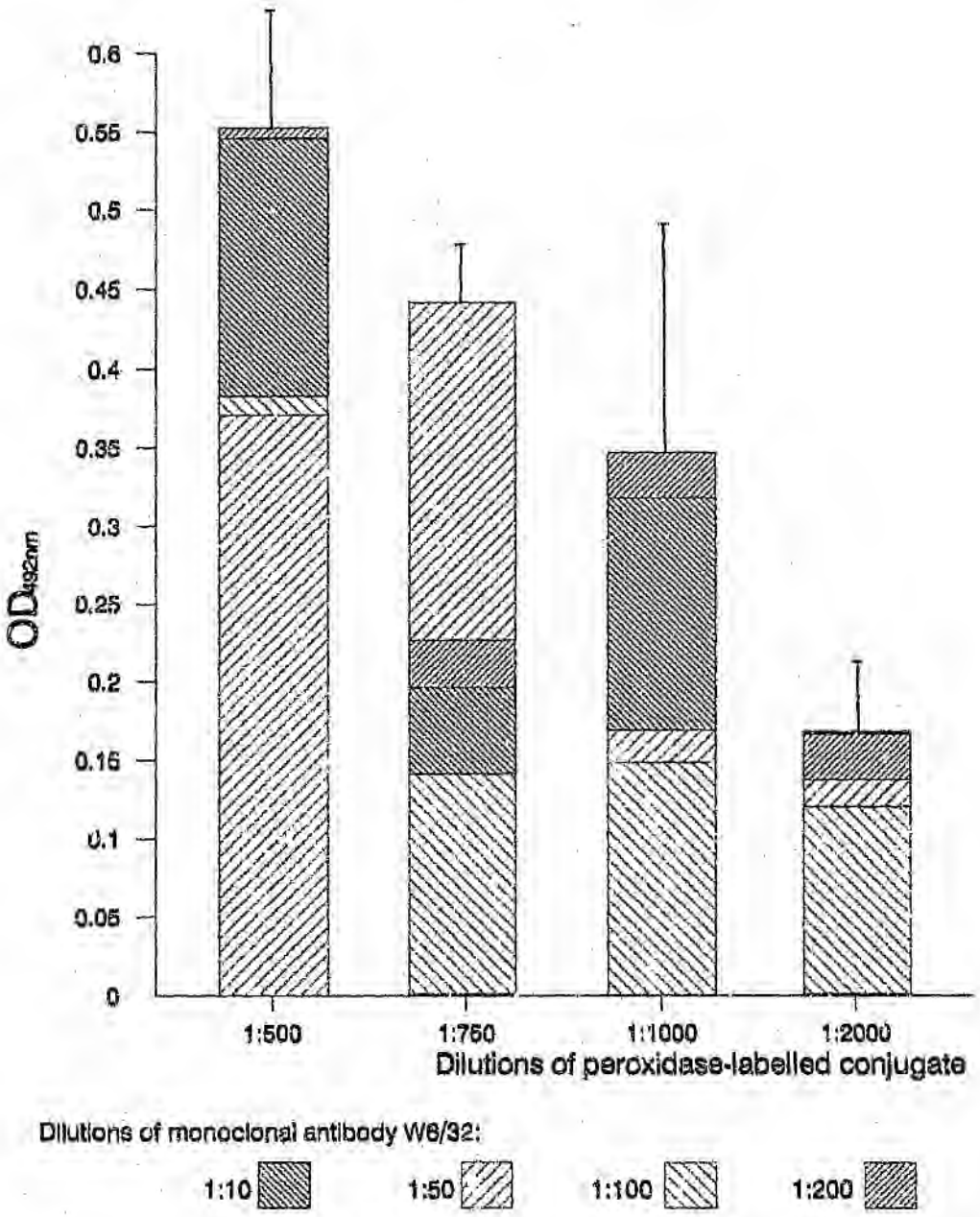


Dilutions of monoclonal antibody W6\32:

1:10 1:50 1:100 1:200

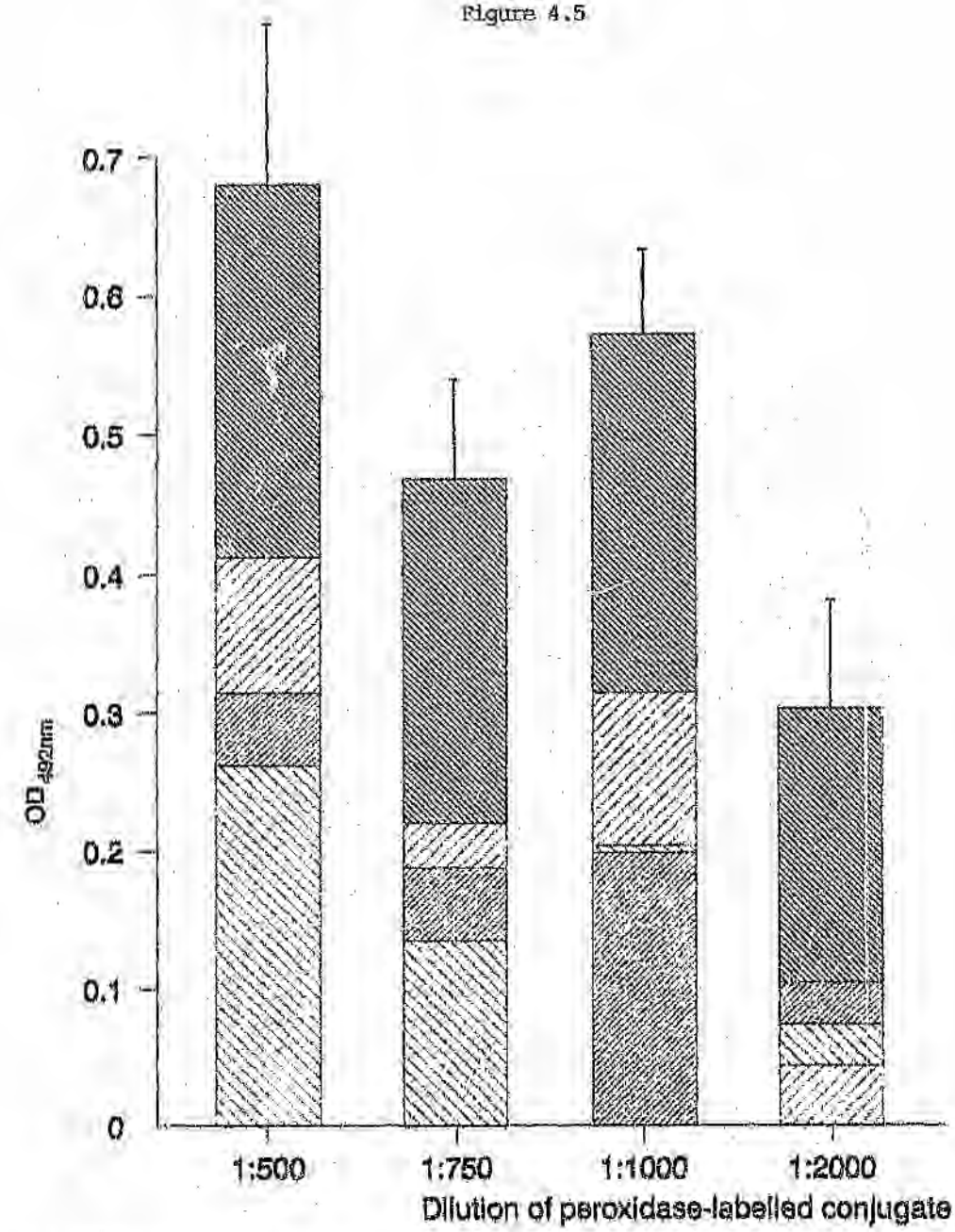
Dose kinetics of monoclonal antibody W6/32 for use in ELISA using the cell line Hep 3B

Figure 4.4



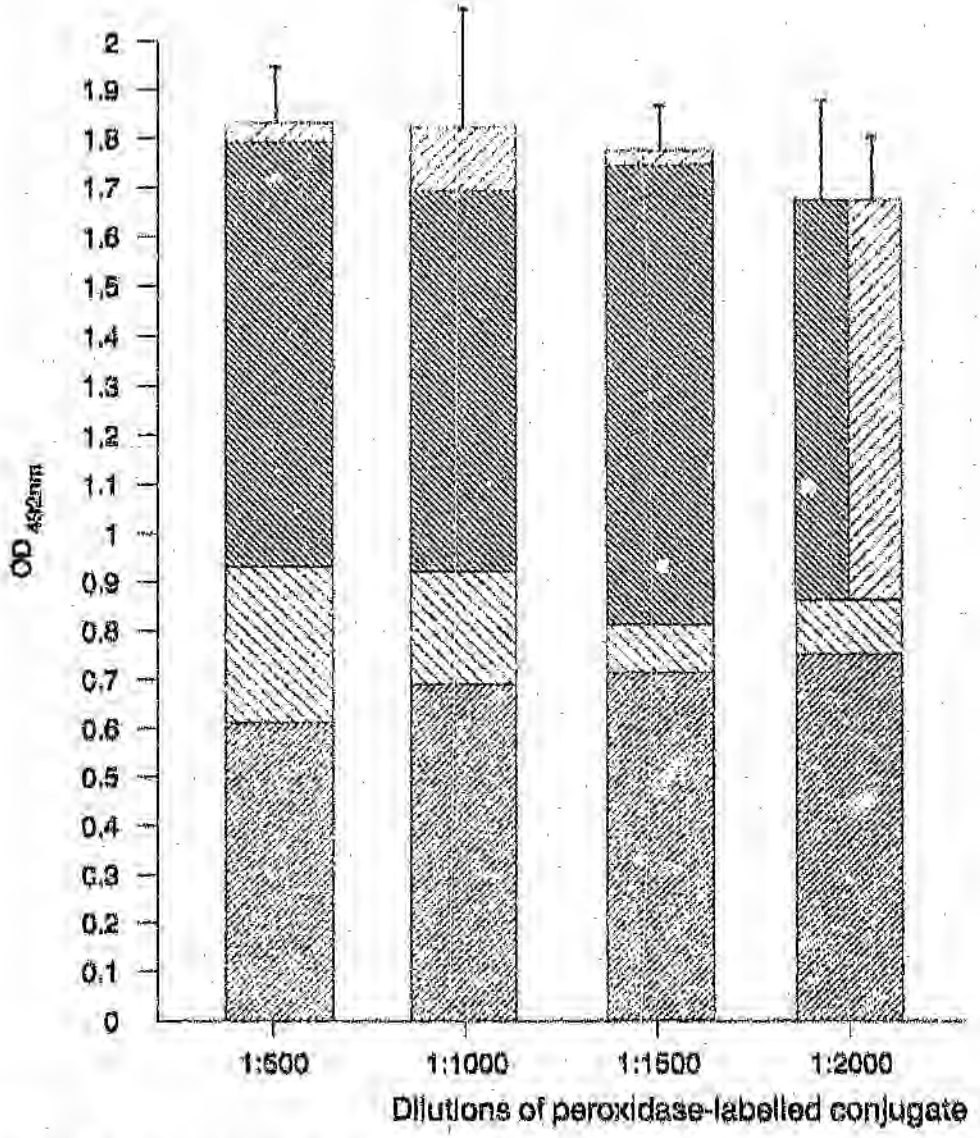
Dose kinetics of the monoclonal antibody W6/32 for use in ELISA using the cell line TONG PHC.

Figure 4.5



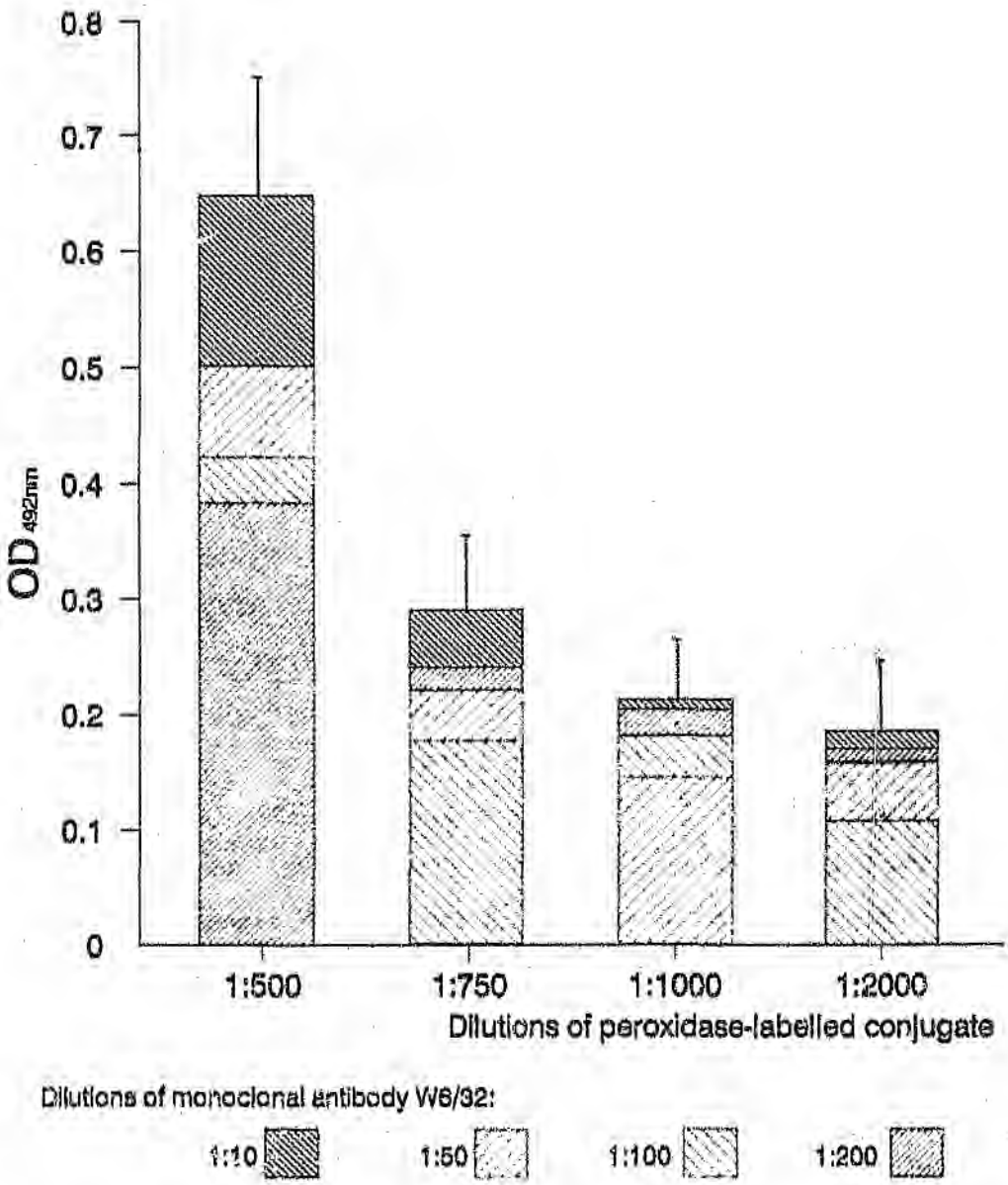
Dose kinetics of the monoclonal antibody W6/32 for use in ELISA using the cell line HA59T/VGH

Figure 4.6



Dose kinetics of the monoclonal antibody W6/32 for use in ELISA using cell line HA22T/VGH

Figure 4.7



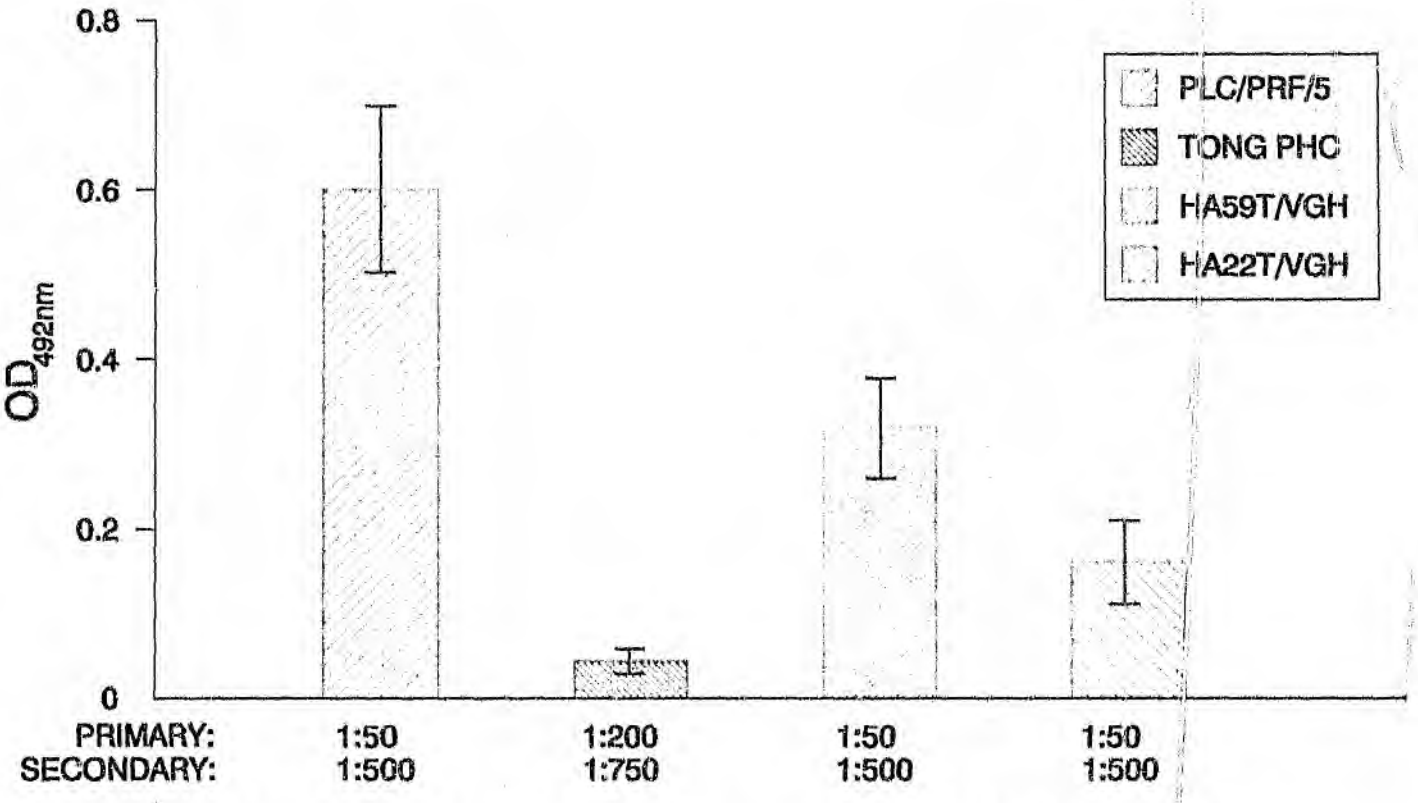
Dose kinetics of the monoclonal antibody W6/32 for use in ELISA using the cell line PLC/PRF/5

former or latter doses of the antibody. In figure 4.3, a dilution of 1:100 of the MOAB W6/32 and a 1:500 dilution of the enzyme-labelled antibody resulted in a non-specific response to the primary antibody being observed. Using the same dilution of the MOAB, the same type of response was observed at a lower dilution of the secondary antibody (1:1000). However when the dilution of the MOAB was 1:200, a dose dependant response was observed as either higher or lower dilutions of the secondary antibody resulted in lowered optical density values (see Fig 4.3). A similar type of dose dependant response of the MOAB W6/32 was observed on the cell line TONG PHC, as shown in Fig. 4.4. The optimal dilutions for W6/32 and the peroxidase-labelled conjugate on this cell line were 1:50 and 1:750 respectively. Higher and lower dilutions of the primary and secondary antibodies resulted in an inconsistent response being observed as measured at an OD of 492 nm. On the cell line PLC/PRF/5 (Fig 4.7), the optimisation of the primary and secondary antibody occurred at dilutions of 1:50 and 1:500 respectively. As shown in Figure 4.7, the Alexander cell line, PLC/PRF/5, appeared to express a decreasing amount of HLA Class I antigen with higher dilutions of the MOAB as measured at an OD of 492 nm. On the HCC continuous cell lines HA22T/VGH and HA59T/VGH, a variable dose response of the primary and secondary antibody was observed (Figs 4.5 and 4.6 respectively). As shown in Figure 4.5, the cell line HA22T/VGH appeared to

exhibit a constant response despite varying antibody dilutions. This cell line also exhibited the highest amount of spontaneous MHC Class I antigen expression as measured at an OD of 492 nm (Fig 4.5). The consistency that was observed in antibody dose response could perhaps be because this cell line exhibited a minimal number of Class I antigenic sites that were easily saturated irrespective of antibody dilution used. In Figure 4.4 the variable dose response observed for the cell line HA22T/VGH is mimicked at all dilutions of primary antibody used. Dose response curves were obtained as a mean of three experiments performed at different times.

The optimal dilutions of the primary antibody, TAL IB5 (anti-HLA-DR) and the peroxidase-labelled conjugated antibody for the HCC cell lines PLC/PRF/5, TONG PHC, HA59T/VGH and HA22T/VGH are shown in Figure 4.8 (results of the Hep 3B, Hep G2, Mahlavu cell lines not shown). As can be seen in Figure 4.8, MHC Class II antigens were spontaneously expressed by PLC/PRF/5, HA59T/VGH and HA22T/VGH cell lines whilst TONG PHC (as well as Hep 3B, Hep G2 and Mahlavu results not shown) only exhibited negligible amounts of MHC Class II antigens spontaneously. The figures 4.9-4.12 demonstrate the doses of the MOAB TAL IB5 required for the cell lines PLC/PRF/5, HA59T/VGH, HA22T/VGH and TONG PHC. Figure 4.9, illustrates that PLC/PRF/5 spontaneously expresses HLA-DR antigens at all

Figure 4.8



OPTIMAL DOSE RESPONSE OF

1)PRIMARY ANTIBODY:ANTI-HLA-DR(TAL-IB5)

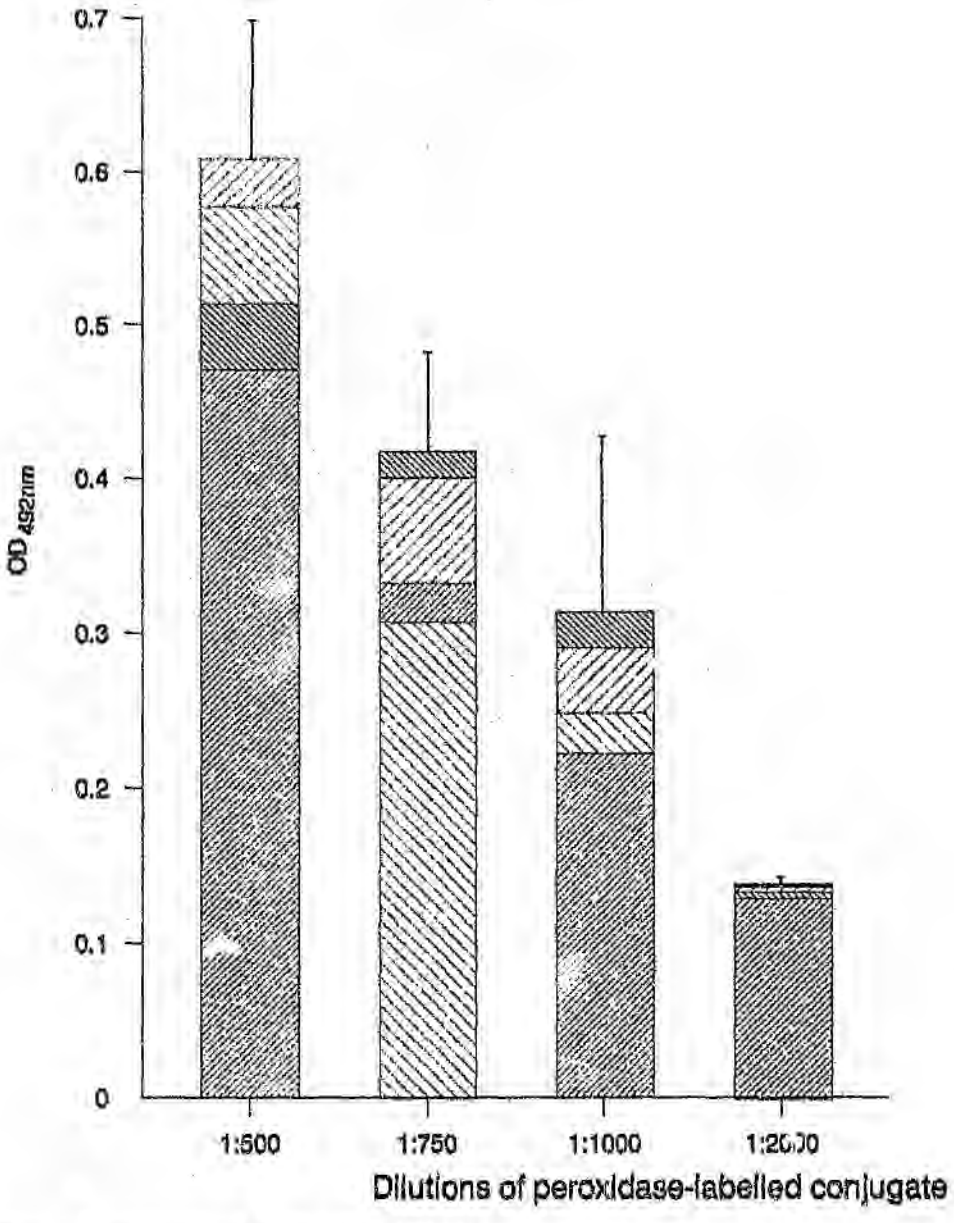
2)SECONDARY ANTIBODY:PEROXIDASE-LABELLED CONJUGATE

doses of the MOAB and enzyme-labelled antibody investigated. Doses of 1:50 and 1:500 of the primary and secondary antibodies respectively were optimal as at these dilutions maximal detection of the MHC antigens occurred.

High standard deviations were observed in Figure 4.10 in the determination of the optimal dose on the HA59T/VGH cell line. This could suggest that a dilution of 1:10 of of the MOAB TAL IB5 was too low experimentally resulting in maximal non-specific interactions of the MOAB with antigenic sites. On the cell line HA22T/VGH, (Fig. 4.11), the optimal dilution of the primary and secondary antibodies required was 1:50 and 1:500 respectively. This dilution of 1:50 of the primary antibody was seen as optimal in contrast to a 1:10 dilution because of the unsatisfactory standard deviation as well as of the cost of using the MOAB at this low dilution. Higher dilutions of the MOAB TAL IB5 resulted in unsatisfactorily large standard deviations.

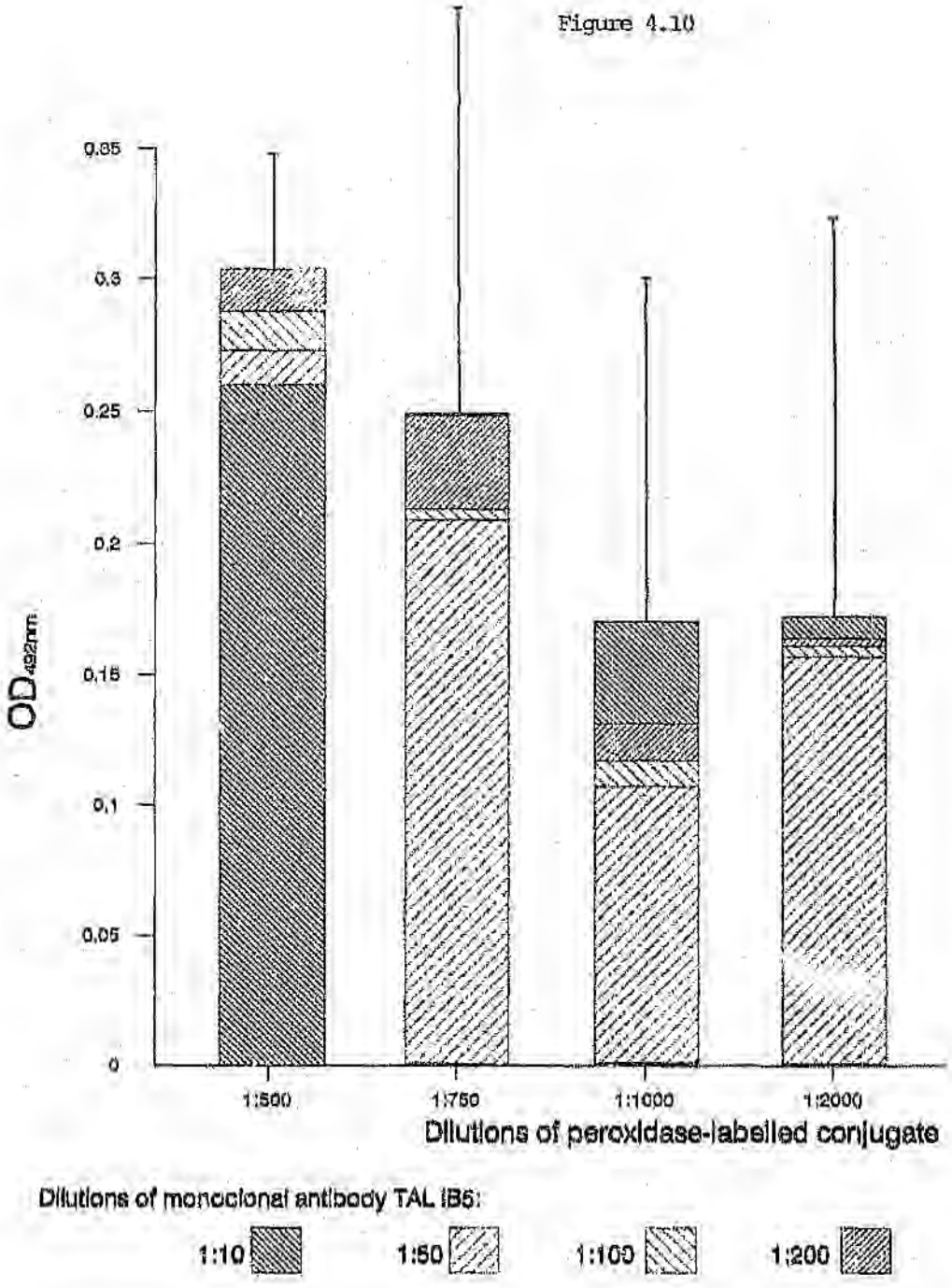
In the cell line TONG PHC (Fig. 4.12), a dilution of the peroxidase-labelled conjugated antibody of 1:500 resulted in non-specific interactions being observed for all dilutions of the primary antibody TAL IB5 used. This dilution of secondary antibody resulted in a false positive

Figure 4.9



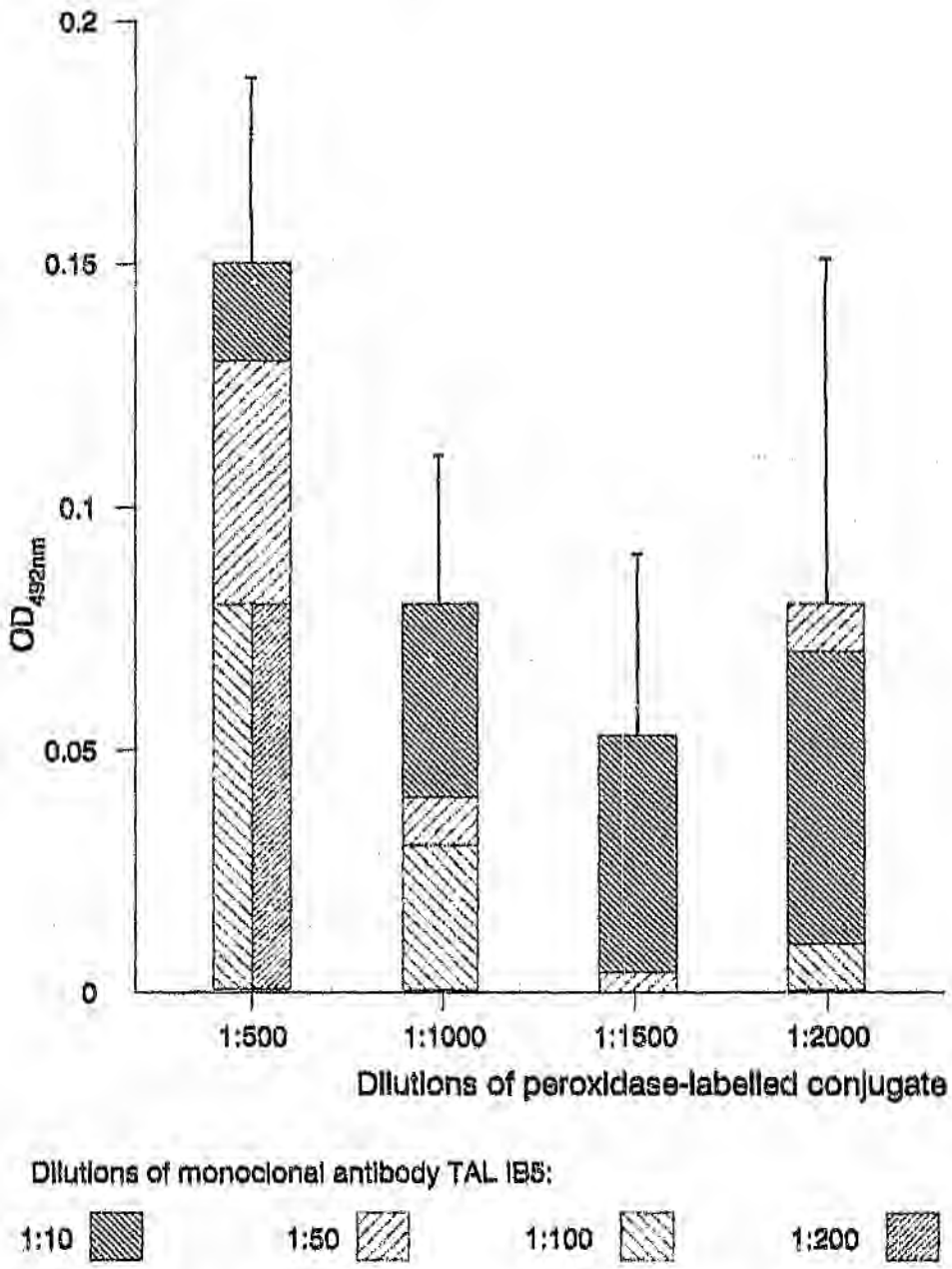
Dose kinetics of the monoclonal antibody TAL IB5 for use in ELISA using the cell line PLC/PRF/5

Figure 4.10



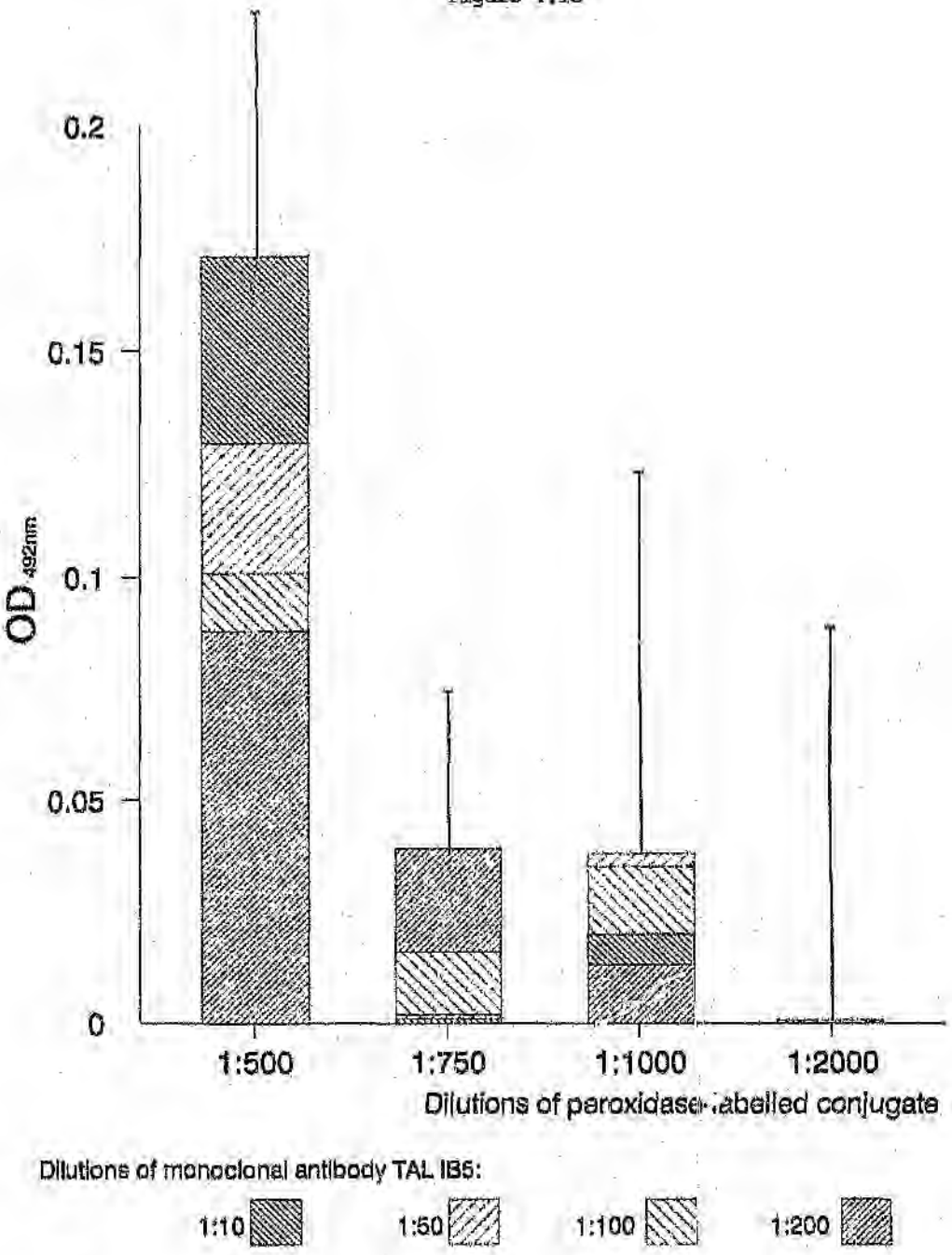
Dose kinetics of the monoclonal antibody TAL IB5 for use in ELISA using the cell line HA59T/VGH

Figure 4.11



Dose kinetics of the monoclonal antibody TAL IB5 for use in ELISA using cell line HA22T/VGH

Figure 4.12



Dose kinetics of the monoclonal antibody TAL IB5 for use in ELISA using the cell line TONG PHC

(Fig. 4.12), since the OD value obtained was higher than the OD value of 0.04 obtained for the negative control cell line K562 (Fig. 4.15). At higher dilutions of the peroxidase-labelled conjugate, the spontaneous expression of HLA-DR antigens as detected by the ELISA decreased dramatically. A dilution of 1:2000 of the secondary antibody detected almost zero amounts of HLA-DR antigens (Fig 4.12).

In order to optimise the d[illegible] of the MOAB directed against ß2 microg[illegible] one set of dose response experiments (mea[illegible] at experiments) was performed using thr[illegible]T/VGH. As seen in Figure 4.13, the use of this [illegible]ted in satisfactory OD values only at a dilution of 1:10. However these OD measurements were coupled with high standard deviations values. Therefore this monoclonal antibody, although product specifications included use for the ELISA, was unsatisfactory for this study. However unavailability and cost of other anti-ß2 microglobulin monoclonal antibodies precluded the use of other commercially available products.

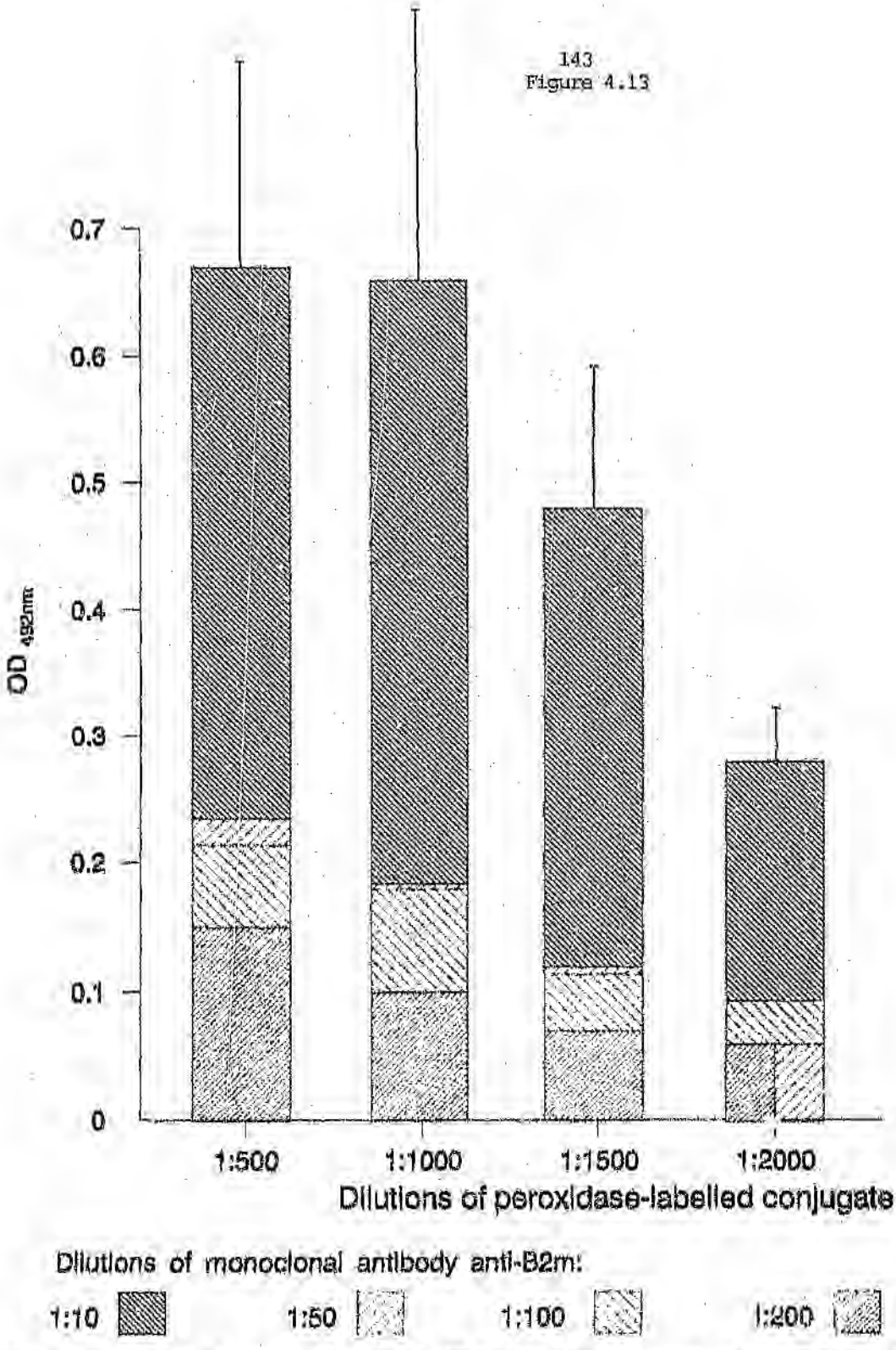
According to the supplier's specifications (results not shown) the optimal dilution of the MOAB directed against the HLA-DR region associated invariant chain, VIC Y1, was 1:1000. This dilution was confirmed by the experiment

performed on the HA22T/VGH cell line using 1:500, 1:750, 1:1000, 1:1500 and 1:2000 dilutions of the primary antibody. The peroxidase-labelled conjugate was used at dilutions of 1:1000, 1:1500, 1:2000 and 1:2500 (Fig. 4.14). When the primary and secondary antibodies were both used at a dilution of 1:1000, the highest optical density measurements were obtained. Higher dilutions of the primary antibodies and secondary antibodies (eg 1:2000 and 1:2500 respectively) resulted in a non-specific response since at these dilutions negligible amounts of the antigen was detected. At lower dilutions (eg 1:750 of the primary and 1:1000 of the secondary antibodies) high values for the standard deviations were obtained implying non-specificity.

4.4.2 DETECTION OF MHC ANTIGENS ON RAJI AND THE K562 CELL LINES.

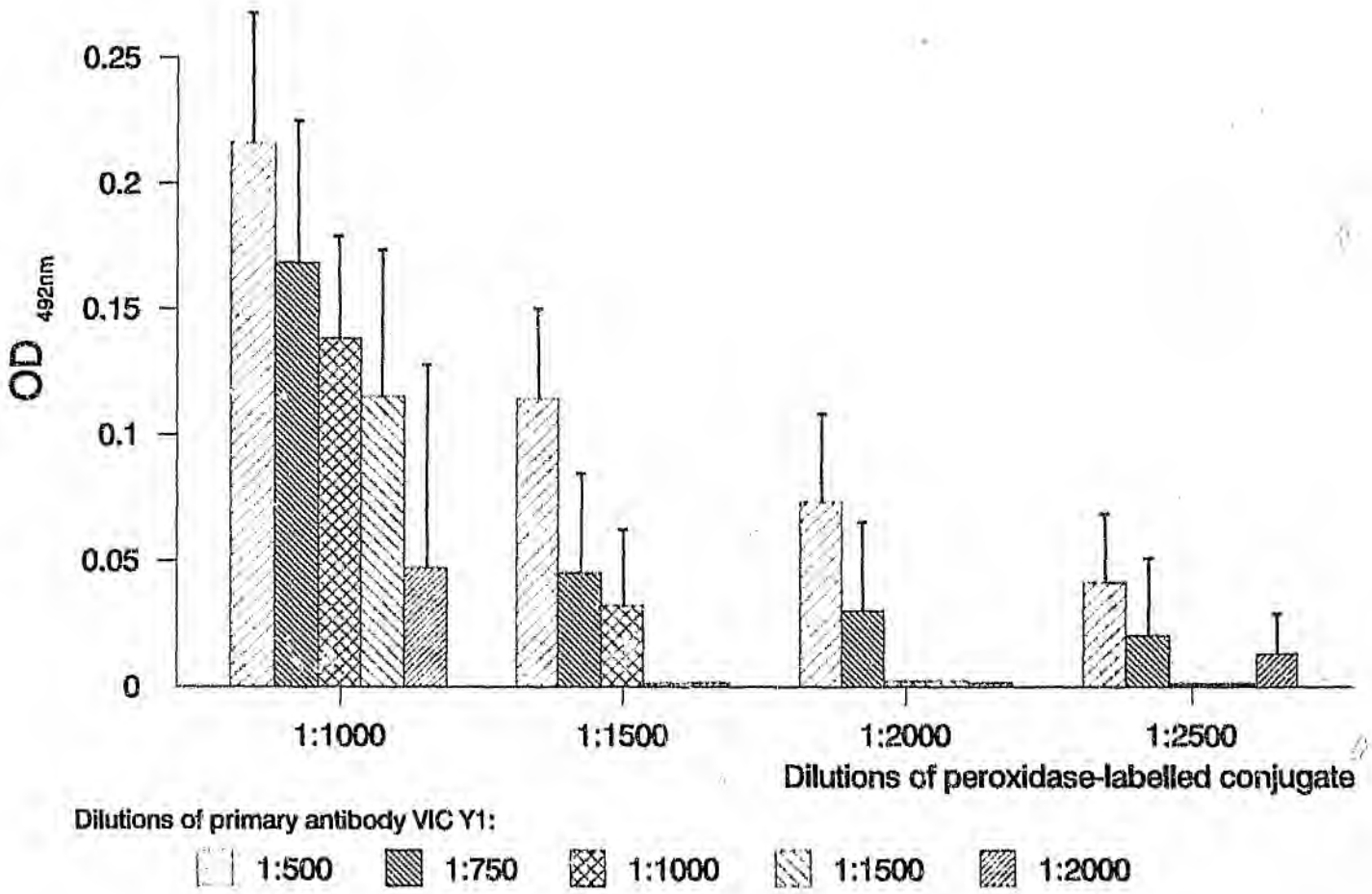
The validity of the assignments of K562 and Raji cells lines as the negative and positive controls respectively were borne out by the optical densities obtained for the detection of MHC surface expression on these cell lines (figures 4.15 and 4.16 respectively). When optimal dilutions of the primary antibodies directed against MHC Class I (W6/32) and Class II (TAL IB5) as well as their respective invariant chains ß2m (anti-ß2m) and Ii (VIC Y1) were used in the assessment of antigen expression on the

Figure 4.13



Dose kinetics of anti-B2m for use in ELISA using cell line HA22T/VGH

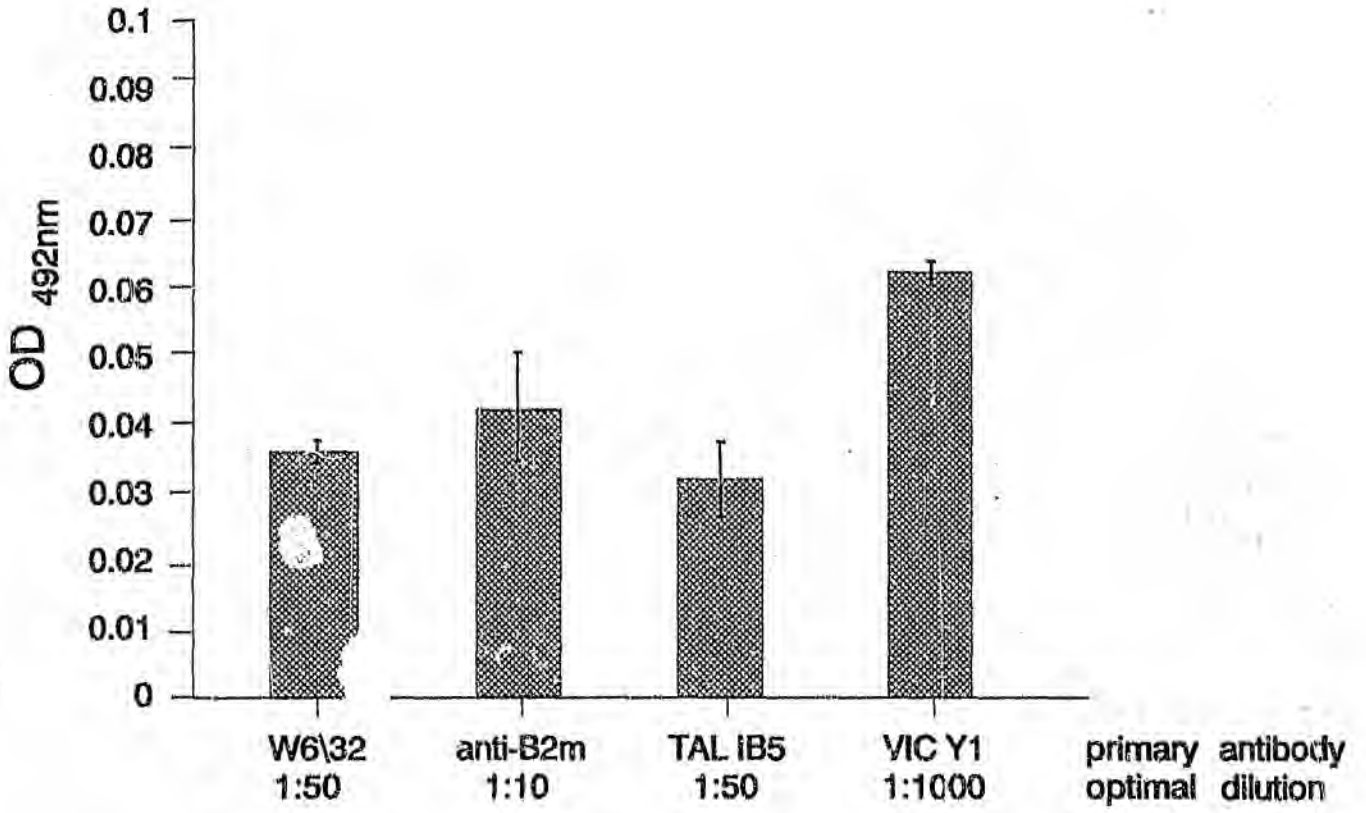
Figure 4.14



Dose kinetics of VIC Y1 for use in ELISA using cell line HA22T/VGH.

erythromyeloid cell line K562 (Fig. 4.15), negligible amounts of MHC antigens were detected at an OD of 492 nm. The optical density value of 0,035 was obtained when detecting HLA-A,B,C, using the MOAB W6/32, and 0,032 OD units when detecting HLA-DR antigens using TAL IB5. Measurement of MHC Class I and II antigens resulted in optical density values of not greater than 0,04 (including standard deviations). For this reason, any optical density value below this value of 0,04 indicated that the particular class of MHC antigen was not spontaneously expressed. Any OD values obtained above the value of 0,04 were interpreted as that the cells spontaneously expressed a particular class of MHC antigens. In the cell line TONG PHC (Figure 4.12) a negligible amount of spontaneous MHC Class II expression as detected by the MOAB TAL 1B5 was observed. The OD value measured at dilutions of 1:200 and 1:750 for the primary and secondary antibodies respectively was 0,04 unit . This finding indicated that TONG PHC did not express MHC Class II antigens spontaneously. Figure 4.16 shows the results obtained for MHC Class I and II as well as their associated invariant chains, β2m and Ii respectively, on the cell line Raji. As observed in figure 4.16, MHC antigens were detected by their respective primary antibodies used at optimised dilutions and were spontaneously expressed in high amounts.

Figure 4.15



ELISA results of control cell line K562 using an optimal dilution of 1:500 of the secondary antibody.

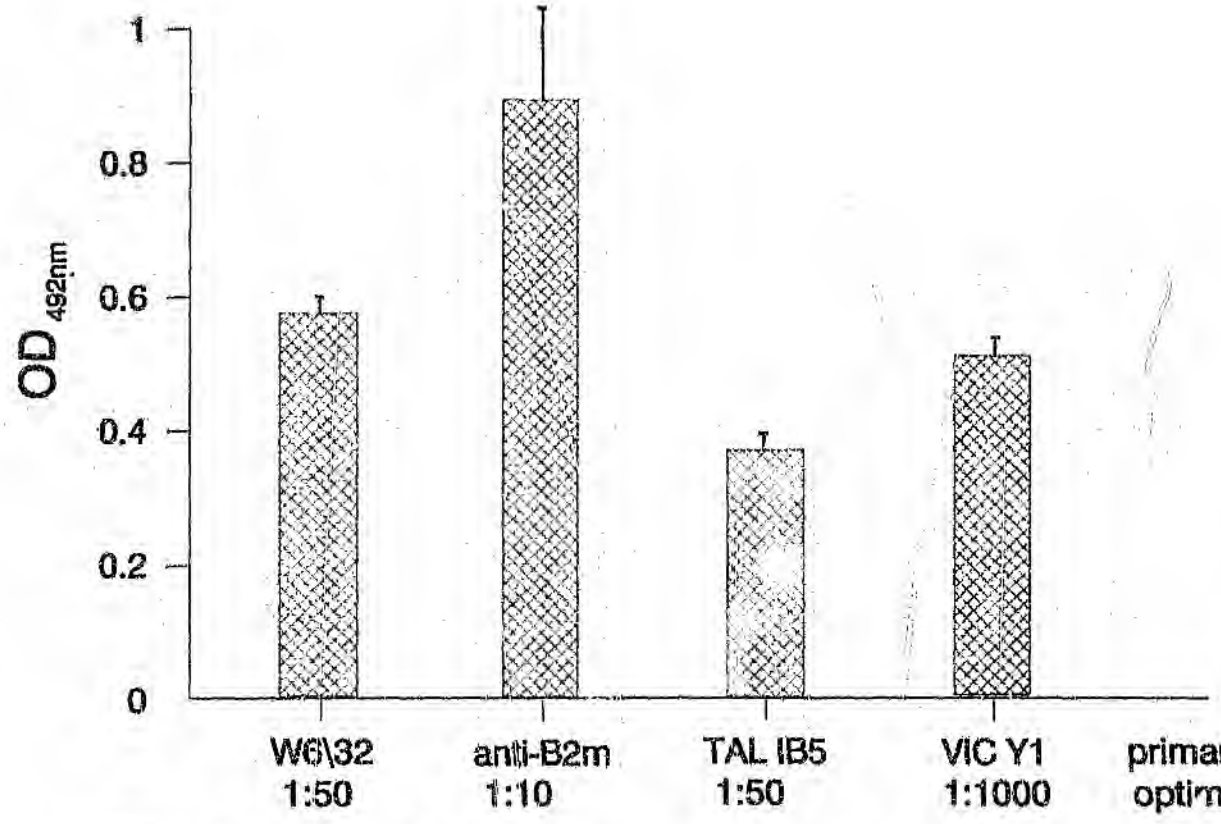


Figure 4.16

ELISA results of control cell line Raji using an optimal dilution of 1:500 of the secondary antibody.

4.4.3 RESULTS OF MHC ANTIGEN EXPRESSION AFTER LYMPHOKINE AND CHEMICAL STIMULATION.

Figures 4.17-4.19 show the responses obtained for the different doses of the lymphokines and chemicals on the HCC cell lines TONG PHC, HA22T/VGH and HA59T/VGH. These figures show responses as measured in OD units at an optimised incubation time of 24 hours. The OD values for stimulated cells (xxxx) are compared with those measured for unstimulated or control cells (////) as seen in figures 4.17-4.19 . The effect of each dose of the lymphokines or chemicals used was determined using MOABs directed against MHC Class I, Class II, ß2m and in some cases, Ii. The results of the effects of the chemicals, sodium butyrate and clofazimine and the lymphokines, interferon-alpha and -gamma are given under separate headings.

4.4.3.1 EFFECTS OF CLOFAZIMINE ON MHC EXPRESSION

Clofazimine was used at the dosages of 0,1, 1, 2ug/ml as well as 0,1, 1, 5 ug/ml. On the cell line HA59T/VGH (fig 4.17) clofazimine did not increase the expression of Class I antigens at any of the dosages investigated. For this class of MHC antigen, the OD values of clofazimine stimulated cells were less than those obtained for the unstimulated or control cells as detected by the MOAB W6/32 (anti-HLA-A,B,C). This result implied an inhibition of MHC

Class I expression when cells of this cell line were incubated with clofazimine. The expression of ß2m was negligibly enhanced after incubation with 0,1 ug/ml but at higher doses of clofazimine, this effect fell away (fig 4.17). The optimal dose of clofazimine for obtaining maximal differentiation between stimulated and unstimulated cells was 1,0 ug/ml when measuring HLA-DR expression. The effect of clofazimine on Ii expression was variable with no significant enhancement of this invariant chain observed (fig 4.17).

In the cell line TONG PHC (Fig 4.18), HLA expression increased with higher doses of clofazimine. Similarly, as in the cell line HA59T/VGH, at lower doses of clofazimine (0,1 ug/ml) no enhancement of HLA-A,B,C was observed in TONG PHC. At higher doses of clofazimine (1 and 2 ug/ml) the differentiation between stimulated and unstimulated cells increased with a 2 ug/ml dose causing a substantial enhancement in MHC Class I expression. The optimal dose of clofazimine for enhanced ß2m expression was 1ug/ml. At this dose, maximal differentiation of stimulated and unstimulated cells was observed when detecting ß2m. Irrespective of the dose of clofazimine used, HLA-DR expression was enhanced in a constant manner (1.5 fold increase in OD units). However, this enhancement was not very marked when compared to that caused by sodium butyrate or gamma-interferon (Fig 4.15).

Figure 4.17

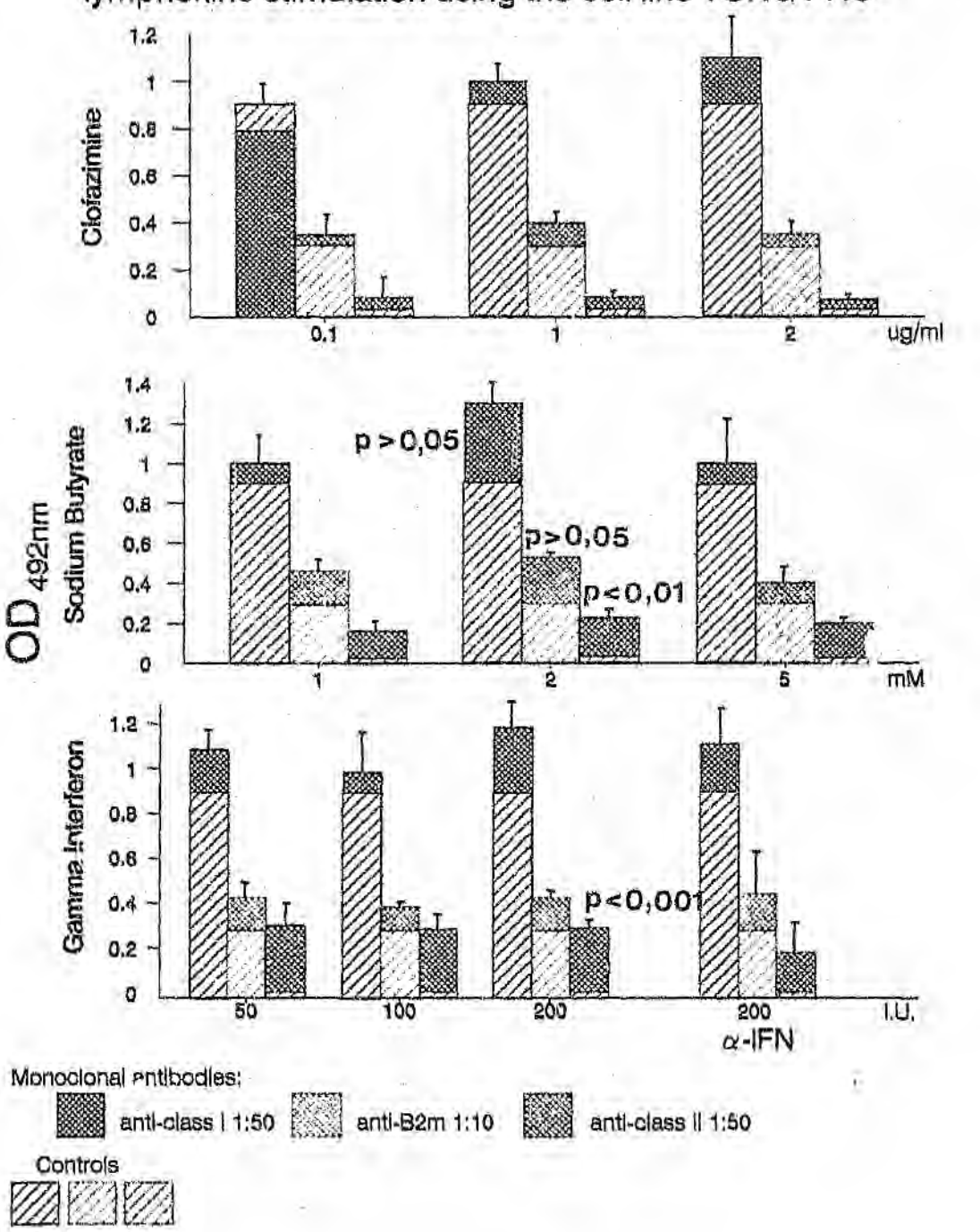

Expression of MHC class I and II antigens following chemical and lymphokine stimulation using the cell line TONG PHC
Clofazimine
1.2
1
0.8
0.6
0.4
0.2
0
0.1
1
2
ug/ml
OD 492nm
Sodium Butyrate
1.4
1.2
1
0.8
0.6
0.4
0.2
0
p > 0,05
p > 0,05
p < 0,01
1
2
5
mM
Gamma Interferon
1.2
1
0.8
0.6
0.4
0.2
0
p < 0,001
50
100
200
200
I.U.
α-IFN
Monoclonal antibodies:
anti-class I 1:50
anti-B2m 1:10
anti-class II 1:50
Controls

Figure 4.18

Expression of MHC class I and II antigens following chemical and lymphokine stimulation using the cell line HA22T/VGH

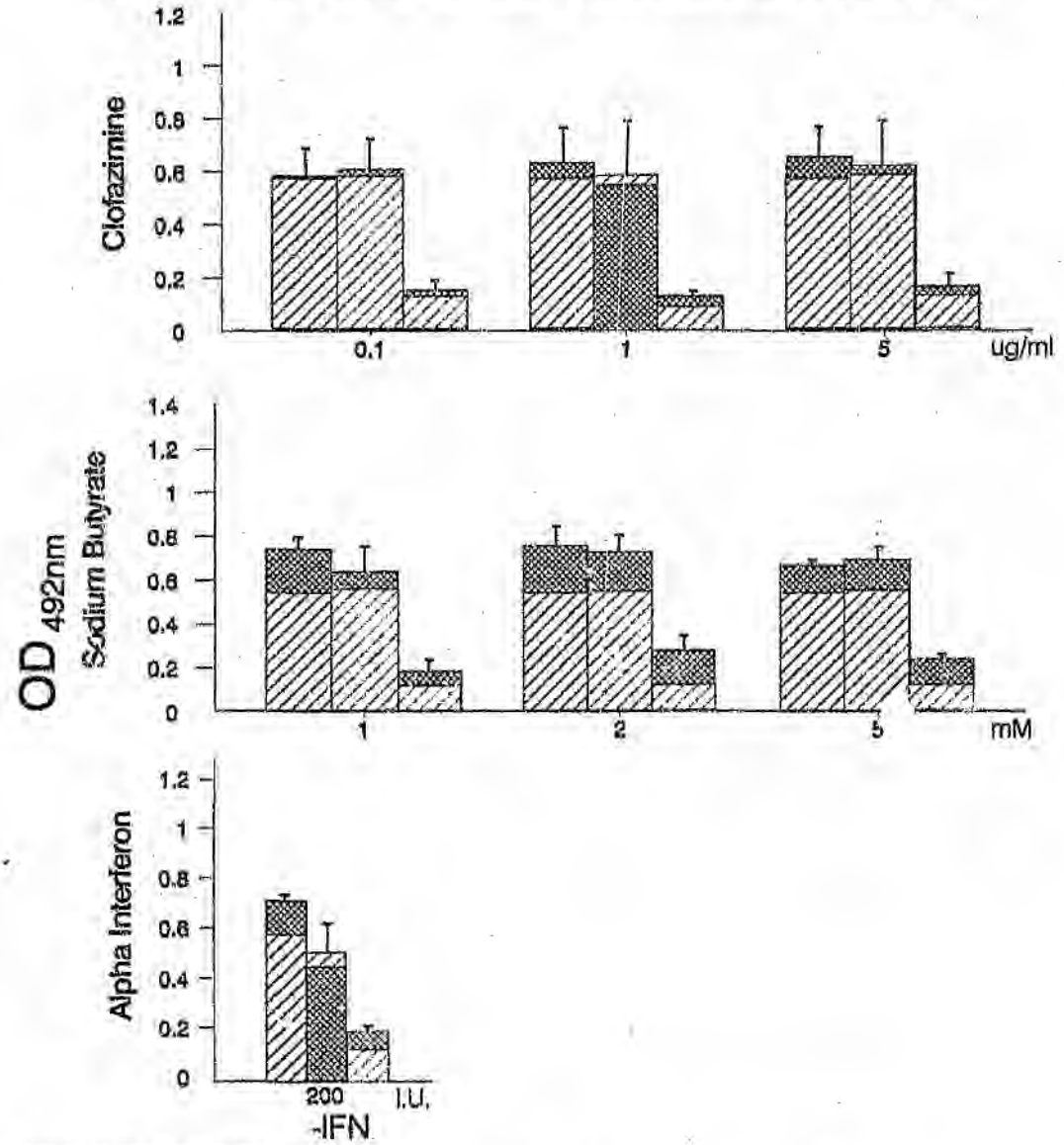
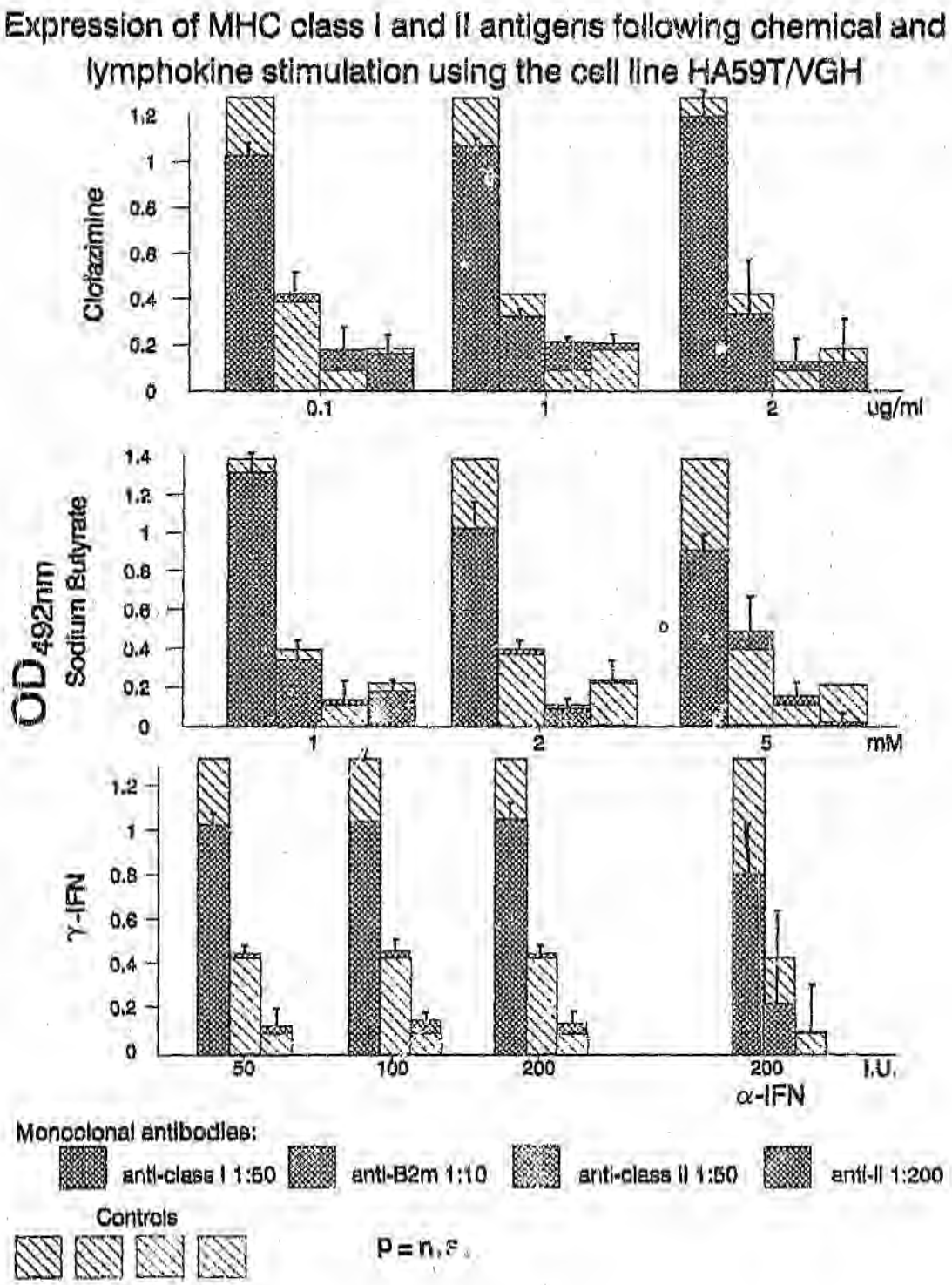


Figure 4.19


Expression of MHC class I and II antigens following chemical and lymphokine stimulation using the cell line HA59T/VGH
Clofazimine
1.2
1
0.8
0.6
0.4
0.2
0
0.1
1
2
ug/ml
OD492nm
Sodium Butyrate
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
5
mM
γ-IFN
1.2
1
0.8
0.6
0.4
0.2
0
50
100
200
200
I.U.
α-IFN
Monoclonal antibodies:
anti-class I 1:50
anti-B2m 1:10
anti-class II 1:50
anti-II 1:200
Controls
p = n.s.

In the cell line HA22T/VGH (Fig 4.19) the effect of clofazimine on MHC expression was not as enhanced (at any dose investigated) when compared to that on the two cell lines discussed above. HLA Class I antigen expression was increased with the highest dose of 5 ug/ml causing a greater enhancement than a 0,1 ug/ml dose. Even at this higher dose, Class I expression was not markedly enhanced. The response of ß2m to this chemical was variable with no consistent enhancement noted. HLA-DR expression was slightly enhanced in HA22T/VGH after incubation with clofazimine with the largest effect of this chemical occurring at a dose of 1ug/ml (fig 4.19).

4.4.3.2 EFFECTS OF SODIUM BUTYRATE ON MHC EXPRESSION

Sodium butyrate was used at dosages of 1,2,5 mMolar. No significant effect of sodium butyrate at any dose was observed on the HCC cell line HA59T/VGH after an optimised incubation time of 24 hours (fig 4.17). HLA-A,B,C expression on stimulated cells appeared to decrease with higher dosages of this chemical when compared to unstimulated cells. The expression of ß2m was not increased at the lower doses investigated however, a dose of 5 mM resulted in an enhancement of ß2m expression. The response of both HLA-DR and Ii to this chemical was variable. A slight enhancement was observed at certain dosages, while a inhibition in these antigens' expression was noted at other dosages (fig 4.17).

The effect of sodium butyrate on MHC expression was most marked in the cell line TONG PHC (Fig 4.18). MHC Class I, Class II and ß2m antigens exhibited large increases in expression. MHC Class I expression was enhanced after incubation with sodium butyrate at all the dosages investigated. The largest enhancement of this antigen's expression occurred at a dose of 2 mM sodium butyrate (fig 4.18). This dose was therefore regarded as optimal. A similiar effect was observed in ß2m expression after incubation with sodium butyrate with the optimal dose occurring in the range of 2 mMolar. HLA-DR expression was markedly enhanced (8 fold increase in OD units) in the TONG PHC cell line after incubation with this chemical. The dose of sodium butyrate that caused the maximal differentiation between stimulated and unstimulated cells was in the range of 2mM. This dose was therefore regarded as optimal in enhancing HLA-DR expression (Fig 4.18).

In the cell line HA22T/VGH (Fig 4.19) incubation of these cells with sodium butyrate resulted in enhanced MHC expression, although this effect was not as marked when compared with that observed in figure 4.18. As in TONG PHC, MHC Class I and ß2m antigen expression was enhanced with the optimal dose occuring in the range of 2mM sodium butyrate (fig 4.19). HLA-DR expression was markedly enhanced in the HA22T/VGH cell line after incubation with sodium butyrate. The optimal dose of sodium butyrate that

resulted in the most marked enhancement of MHC Class II antigen expression was in the range of 2mMolar (fig 4.19). This is well within the physiological concentration of sodium butyrate.

4.4.3.3 EFFECTS OF INTERFERON-GAMMA AND -ALPHA ON MHC EXPRESSION

The effect of gamma interferon on MHC expression was investigated at dosages of 50, 100, 200 IU/ml after an optimised time of 24 hours. Recombinant-IFN -alpha was used at a constant dose of 200 IU/ml at an optimised incubation time of 24 hours. This lymphokine was used as a control to assess the specificity of the effects of r-IFN-gamma. That is, whether or not the resulting enhancement of MHC expression was because of r-IFN-gamma.

In the cell line HA5T/VGH (Fig 4.17) no effect on MHC Class I expression was noted after these cells were incubated with both interferons used in this study. There was no difference in the lack of HLA-A,B,C expression at all the dosages of r-IFN-gamma investigated. A lower expression of these antigens was observed when cells of HA59T/VGH were incubated with r-IFN-alpha. A negligible enhancement of B2m expression was observed at all dosages of r-IFN-gamma used. HLA-DR expression was enhanced, but only slightly when compared to that observed in TONG PHC (Fig 4.18) after incubation with r-IFN-gamma. The optimal dose of

r-IFN-gamma for this effect to be most marked was 100 IU/ml. No enhancement of MHC Class II antigen expression in HA59T/VGH was observed after incubation with 200 IU/ml r-IFN-alpha at a time of 24 hours (fig 4.17).

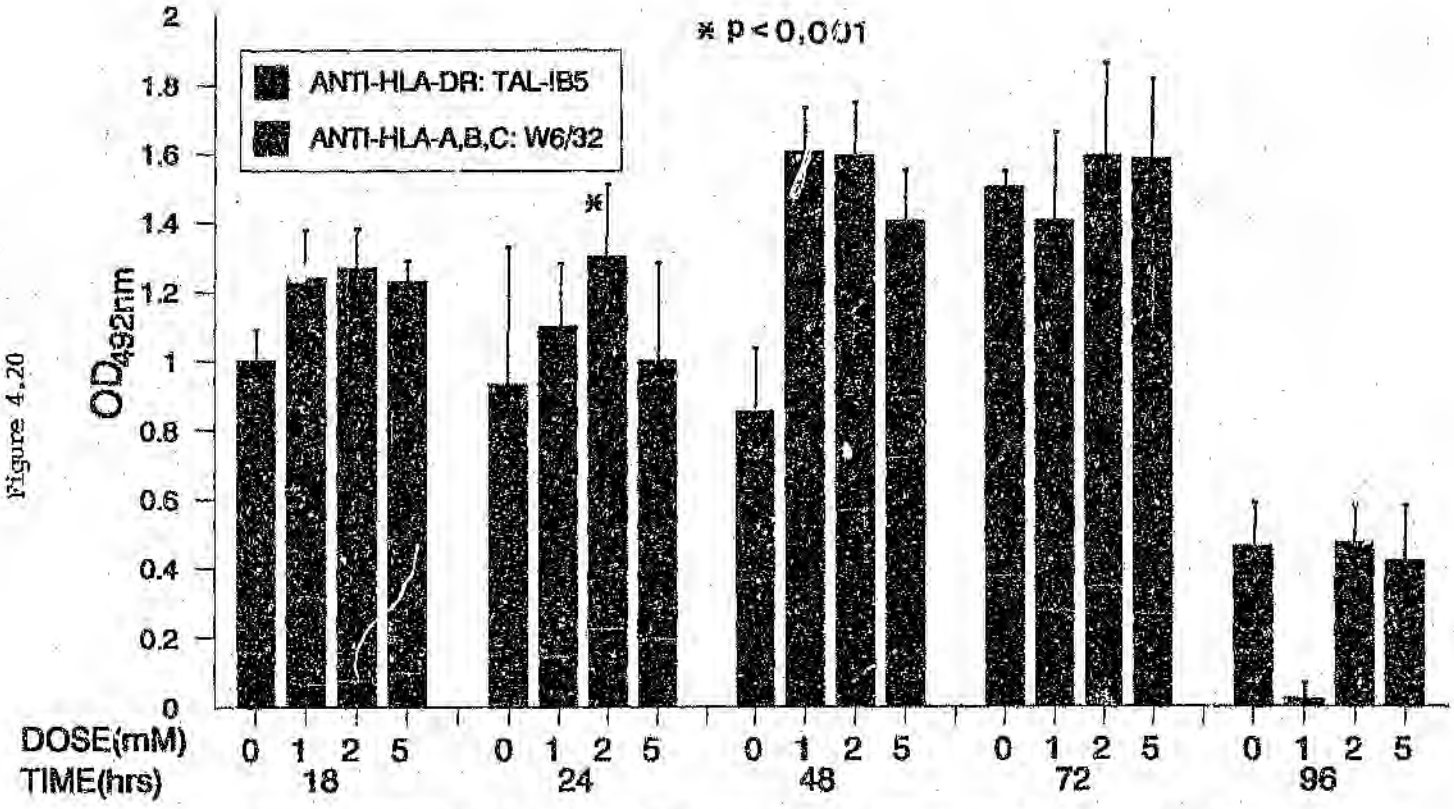
The most noticeable feature observed in the TONG PHC cell line (Fig 4.18) after incubation with r-IFN-gamma was the substantial increase in HLA-DR expression (a 12-fold increase in OD values). This enhancement of HLA-DR expression were raised when any of the doses of r-IFN-gamma used. A slightly less marked enhancement (8 fold increase in OD units) of MHC Class II expression was observed after incubation with r-IFN-alpha. MHC Class I expression was enhanced after incubation with r-IFN-gamma at all dosages investigated. The most marked increase in HLA-A,B,C expression occurred at 200 IU/ml of r-IFN-gamma. This dosage was therefore regarded as optimal. The effect of r-IFN-alpha on MHC Class I expression was almost mirrored by that observed at a dosage of 50 IU/ml of r-IFN-gamma. That is, there was no difference in the enhancement of MHC Class I expression after incubation with r-IFN-alpha or IFN-gamma. A similar effect was observed for ß2m expression. The enhancement of this antigen's expression was larger after incubation with r-IFN-alpha than at any dosage of IFN-gamma investigated.

Incubation with 200 IU/ml of r-IFN-alpha resulted in enhanced MHC Class I expression (2.6 fold increase in OD units) in the HA22T/VGH cell line (Fig 4.19). However, no enhancement of ß2m expression after incubation with this lymphokine for 24 hours was observed in this cell line. HLA-DR expression was enhanced after a 24 hour incubation with 200 IU/ml of this lymphokine when compared to unstimulated cells.

4.4.3.4 THE EFFECTS OF LYMPHOKINE AND CHEMICAL INCUBATION TIMES ON HLA EXPRESSION.

Time kinetics were performed at 4, 18, 24, 48, 72 and 96 hours using lymphokines r-IFN-alpha and -gamma and chemicals clofazimine and sodium butyrate. Figure 4.20 depicts the time response curve obtained for sodium butyrate using dosages of 0, 1, 2,5 mM on the representative cell line TONG PHC. At four and 96 hours a nonspecific response was observed. Incubation times of 48 and 72 hours resulted in a non-specific increase of HLA-DR antigen expression on cells that had not been incubated with sodium butyrate (ie control cells). Based on the results obtained for MHC antigen expression in the control cell line, K562 (Fig 4.15), TONG PHC expressed negligible amounts of MHC Class II spontaneously (Fig 4.11). The incubation time of 24 hours resulted in the most specific response of control (unstimulated) cells, that is negligible amounts of HLA-DR expression were observed on

Figure 4.20



TIME-RESPONSE CURVE OF SODIUM BUTYRATE
using the cell line TONG PHC

control cells. This time period was therefore chosen as the optimal incubation time for the maximal stimulation of the cells. Similar types of time response experiments were performed using clofazimine and r-IFN-alpha and -gamma. The same incubation time of 24 hours was observed .

4.5 DISCUSSION

Shimonkevitz et al (1983) demonstrated, that fixation of antigen-presenting cells with formaldehyde did not impair their ability to present antigen. Similarly, Fukasato et al (1988) demonstrated that fixation had no significant effect on the reactivity of cell surface antigens on PLC/PRF/5 cells to monoclonal and polyclonal antibodies in the ELISA. For the above reasons and because the ELISA protocol had been previously standardised in our laboratory, the ELISA was performed on fixed cells using paraformaldehyde as the fixative.

All assays were performed at least three times and it was observed that intra-assay variation of triplicate determination was low while the intra-assay or day-to-day variation was considerable. This may account for the large values obtained for the standard deviations in Figures 4.10 and 4.13. The monoclonal antibodies proved to be specific and sensitive in the detection of the non-polymorphic regions of MHC Class I and II antigens and their respective

associated invariant chains. The monoclonal antibody directed against ß2m, however, proved to be sensitive only at lower dilutions ie. 1:10 which is unsatisfactory in terms of cost.

Notwithstanding, the modified ELISA was able to quantitate cell surface MHC molecules in a simple, specific and sensitive manner (Fukasato et al 1988). Fukasato et al (1988) stated that their results obtained using ELISA as a method of detecting MHC surface antigens in the cell line PLC/PRF/5 correlated well with those obtained in flow cytometric analysis. These authors found that the Alexander cell line did not spontaneously express MHC Class II antigens.

Results obtained using the ELISA as a method of detecting surface MHC antigens correlate with those obtained from the immunofluorescence assay. MHC Class I antigens were spontaneously expressed in the HCC cell lines: PLC/PRF/5; HA22T/VGH; HA59T/VGH; Hep 3B; Hep G2; Mahlavu and TONG PHC using both the ELISA (Fig 4.2 and Fig 4.13) and the IIF assay. MHC Class II antigens were not expressed spontaneously on the following HCC cell lines: TONG PHC, Hep 3B, Hep G2, Mahlavu (Fig 4.8) using both the ELISA and IIF technique as methods of detection. Spontaneous expression of MHC Class II antigens was observed on the

HA22T/VGH, HA59T/VGH and Alexander cell line as detected by the ELISA (Fig 4.8). No spontaneous expression of HLA-DR was noted on PLC/PRF/5 using the IIF assay and flow cytometric analysis. Franco et al (1988) detected low amount of MHC Class antigens on Alexander cells but Sung et al (1989) and Krief et al (1988) found negligible amounts of spontaneous MHC expression on PLC/PRF/5 using an IIF assay. In this study, comparatively high levels of spontaneous Class II antigens were detected using the ELISA. This difference in results could possibly be due to the increased sensitivity of the ELISA compared with IIF staining or different generations of the cell line being used.

The conditions for optimising the detection of MHC expression on the HCC cell lines were achieved by a series of dose response experiments. In Figures 4.3-4.7 and 4.9-4.12, the optical density values obtained for the various dilutions of primary and secondary antibodies are shown. A dilution was chosen as optimal when 1) it showed the highest OD value, 2) this high OD value obtained was specific ie a standard deviation of not more than 0,15 units was obtained 3) the background non-specific staining was not more than 0,01 OD units and 4) the cost of using the antibody at this dilution was not too high. From these experiments the optimal doses of the primary antibody W6/32 (anti-HLA-A,B,C) as shown in Figures 4.3-4.7, and the

monoclonal antibody TAL IB5 (anti-HLA-DR) as shown in Figures 4.9-4.12 were determined. The optimal dilution of the primary antibodies directed against the associated invariant chains, VIC Y1 (anti-Ii) and anti-ß2m was determined as shown in Fig 4.13 and 4.14 respectively. These two figures show the dose response experiments performed on the representative HCC cell line, HA22T/VGH. The optimal dilutions of the secondary enzyme-labelled conjugated antibody were also determined from these experiments. These dilutions were used for all further experiments.

The validity of the assignment of positive and negative control given to the Raji and K562 cell lines respectively was confirmed by the ELISA (Fig 4.16 and 4.17). MHC antigens were detected on these cell lines using previously optimised dilutions of the primary and secondary antibodies. Raji expressed MHC antigens spontaneously while K562 did not express these antigens.

The responses to the chemical, clofazimine, were different among the cell lines used in these experiments. Results obtained when clofazimine was used to assess its effects on MHC Class I expression in the cell lines HA22T/VGH, HA59T/VGH and TONG PHC (Fig 4.17-4.19) indicated that clofazimine enhances at higher (>5 ug/ml) than lower (0,1 ug/ml) concentration.

When detecting MHC Class II antigens after incubation with clofazimine, the cell lines investigated exhibited different responses (Fig 4.17-4.19). In the cell line HA59T/VGH (Fig 4.17), enhancement of MHC Class II expression was dependant on the dose of clofazimine investigated. The dose of 1 ug/ml resulted in maximal differentiation between stimulated and unstimulated cells. A similar result was observed in HA22T/VGH (Fig 4.18). In the cell line TONG PHC, the enhancement of MHC Class II expression did not depend on the dose of this chemical investigated. Clofazimine has been shown to enhance HLA-DR expression on the surface of macrophages after their priming with PPD (A Wadee-personal communication). The method of detecting these surface antigens was the ELISA. No accounts of MHC enhancement on cells in culture after incubation with clofazimine has been reported.

The physiological concentration of sodium butyrate is 2 mM (Miller et al 1987). Although reports have indicated that results with this cell differentiation inducer have been observed at concentrations of up to 10 mM (Leder & Leder 1975, de Haan et al 1986). The results of this study indicate that sodium butyrate achieves maximal enhancement of HLA expression at its physiological concentration. In Figure 4.18, incubation of sodium butyrate with cells of TONG PHC resulted in enhancement of MHC Class I, Class II

and ß2m expression which was dependant on the dose used. The same effect, although less marked, was observed in the cell line HA22T/VGH (Fig 4.19). No result indicating enhancement was obtained after incubating HA59T/VGH cells with any dose of sodium butyrate (Fig 4.17). Kaneko et al (1990) and Nakagawa et al (1985) investigated alterations in gene expression and differentiation states in the Chang and PLC/PRF/5 cell lines respectively. Both these groups of workers used 1 mM sodium butyrate in their experiments. However, there was no literature account of the effects of this chemical on MHC expression on HCC cell lines. Sutherland et al (1985) reported the stimulation of MHC Class I expression in the K562 erythromyeloid cell line after incubation with sodium butyrate. In their experiments, sodium butyrate achieved maximal enhancement of HLA expression at a dose of 1,5 mM.

Fukusato et al (1988) and Yoshioka et al (1989) demonstrated enhanced expression of HLA Class I antigens on PLC/PRF/5, Chang and SK-Hep cells after reaction with 100 IU/ml of IFN-gamma for 24 hours. These workers used the ELISA as a method of detecting MHC antigens. However, Fukusato et al (1988) and Krief et al (1988) were not able to induce HLA Class II antigens with r-IFN-gamma on the more differented PLC/PRF/5 as well as Hep G2 and Hep 3B (Krief et al 1988) cell lines in spite of the use of optimised experimental conditions. These studies employed

an ELISA (Fukasato et al 1988) and flow cytometry (Krief et al 1988) as methods of detecting MHC antigens. Franco et al (1988) observed that r-IFN-gamma was capable of augmenting the expression of MHC Class II antigens on Alexander cells using an indirect immunofluorescence assay. Krief et al (1988) observed that the level of HLA-DR-expression was inversely related to the degree of cellular differentiation of the cell line. Ücer et al (1985) reported that sensitivity to r-IFN-gamma depended on the number of receptors with respect to enhancement of MHC Class II expression.

In the poorly differentiated HCC cell line HA22T/VGH Krief et al (1988) observed that 10 IU/ml of glycosylated r-IFN-gamma was sufficient to induce 100% HLA-DR expression in this line as measured by flow cytometry. The present study did not investigate the effect of r-IFN-gamma on the cell line HA22T/VGH, however the results obtained after r-IFN-alpha stimulation showed a minimal enhancement of MHC expression (Fig 4.19). There have been no previous reports on the enhancement of HLA expression by r-IFN-gamma on the HCC cell lines TONG PHC and HA59T/VGH. In the cell line TONG PHC, which is defined as a moderately differentiated cell line (Lin et al 1984), HLA-DR expression was substantially enhanced after incubation with both r-IFN-gamma and -alpha (Fig 4.18). This result implied that this effect was because of the general action of these

lymphokines (Fig 4.18). The results of the present study indicated that HA59T/VGH exhibited a slightly enhanced expression of MHC Class II antigens when exposed to 100 IU/ml of r-IFN-gamma for 24 hours.

No assessment of the effect of the chemicals and lymphokines investigated on the K562 cell line was determined in the present study. Incubation with sodium butyrate together with r-IFN-gamma resulted in the stimulation of MHC Class I expression in the K562 cell line (Sutherland et al 1985). Such types of experiments, using the K562 cell line would confirm the specificity of these agents on the enhancement of MHC expression.

CHAPTER 5

GENERAL DISCUSSION

Each tumour system is unique. The level of specific MHC products on tumour cells is one of several characteristics that determine the tumourigenic and metastatic potential of cells (Gopas et al 1989). The different MHC Class I and II products are regarded as independent entities since each locus or even sublocus plays a different role in the regulation of the immune system and cell-cell recognition (Elliott et al 1989). For these reasons, it is difficult to determine the role of MHC Class I expression in malignancy. Many of the hypotheses relating Class I expression, oncogenicity and metastatic potential have arisen from well-described murine models (Gopas et al 1989). Although the etiology of experimental animal tumours is important in determining whether MHC expression influences tumourigenicity or not, the relationship between HLA Class I expression and malignancy is still not firmly established in human (Gopas et al 1989).

A vast literature exists describing the expression of HLA-A,B,C antigens in human malignancy. However, there are several problems interpreting the clinical data. Firstly,

because of the qualitative, subjective nature of the techniques used (eg IIF, PAP staining) absolute comparisons among the different reports are difficult. Secondly, there have been few studies in which the monoclonal antibodies employed distinguished among HLA-A,B,C subregion products. This does not allow examination or detection of noncoordinate expression of HLA Class I genes in any haplotype (Elliott et al 1989). Moreover, since the techniques used are non-quantitative with undefined sensitivity levels, expression of trace levels of MHC antigens that could be functionally relevant may be below the threshold of detectability (Elliott et al 1989). Masking of the membrane-bound HLA antigens by sialic acid glycoconjugates could result in quantitative differences of MHC expression being observed (Gopas et al 1989). Finally, very few of these clinical data have been analysed in a statistically rigorous manner (Elliott et al 1989).

The status of HLA-A,B,C antigen expression in hepatocellular carcinoma (HCC) is controversial (See section 1.6). To date there is limited information available on MHC expression in HCC as a solid tumour or on cell lines (Natali et al 1981, Fukasato et al 1986, Franco et al 1988). The results of Paterson et al (1988) describing HLA expression on solid tumours suggest that malignant transformation of hepatocytes is characterised

by expression of Class I antigens. These antigens may be enhanced or acquired de novo (Tukasato et al 1986, Paterson et al 1988). The clinical relevance of this is still uncertain and requires further investigation. Paterson and colleagues suggest that MHC Class I expression in solid HCC tumours may be a contributing factor to the limited metastatic potential of HCC with Class I expression restricting extensive intrahepatic tumour spread.

In colorectal carcinomas, a loss of HLA Class I expression is observed when compared with non-neoplastic epithelium (Momburg et al 1986, Daar et al 1982, van den Ingh et al 1987). In a study of colorectal carcinoma, Momburg et al (1986) observed a significant correlation between loss of MHC Class I and decreasing degree of differentiation. Metastatic tumours were found to be different in their MHC phenotype when compared with the primary tumour (Momburg et al 1986).

Locus-specific Class II (HLA-DR,DP,DQ) molecules can be readily distinguished by MOABs against nonpolymorphic determinants. Differential expression of DR,DP,DQ molecules have been observed on gastric carcinoma as well as mammary carcinoma (Möller et al 1989).

Van Vreeswijk et al (1988) observed a relationship between the metastatic potential of melanomas and the differential expression of HLA-DP,DQ,DR antigens. Van Duinen examined the differential expression of MHC Class II antigens on metastatic compared with primary melanomas. These workers observed a higher percentage of expression of HLA-DR,DP but not DQ antigens in metastatic than in primary melanomas (van Duinen et al 1988). In addition, van Duinen found that a relationship existed between MHC Class I and MHC Class II levels on melanomas and patient survival.

Paterson et al (1988) observed Class II antigen expression in nearly half of the cases of their series of solid HCC tumours. In these tissues, staining displayed a heterogenous pattern of MHC Class II antigens. These antigens were always associated with Ii display, which was far more intensely staining (Paterson et al 1988). However, there have been few, if any other investigations to date on HLA Class II expression on solid HCC tumours. It has been suggested that the ability to display Class II antigens in response to appropriate stimuli may be constitutive to all cells and that it is the failure to express antigen by a percentage of tumours which represents the aberrant state (Moore and Ghosh 1987). Most investigations being performed on HCC cell lines must still be confirmed established in humans (Gopas et al 1989).

Despite its poor sensitivity and lack of subjectivity, the IFF assay is still a valuable tool in the qualitative assessment of MHC expression (Elliott et al 1989). In this study, IIF was performed in order to determine whether or not the cell line expressed surface MHC. It was not employed as a quantitative assay.

Evaluation of MHC surface expression by IIF by the present study indicated that all HCC cell lines investigated exhibited spontaneous MHC Class I antigen expression. Only two poorly differentiated cell lines, HA22T/VGH and HA59T/VGH, spontaneously expressed MHC Class II products. The IIF assay also validated the use of the Raji and K562 as positive and negative control cell lines respectively.

The IIF assay is rapid, safe and sensitive (Bouchiex et al 1985). IIF requires relatively little and unsophisticated equipment and is inexpensive. By contrast, the radioimmunoassay initiated by Berson et al (1956) requires harmful and expensive nuclides. It is therefore sub-optimal as compared to IIF staining. Peroxidase anti-peroxidase staining is similar in its benefits to the IIF assay and is more sensitive, although it is not as rapid, but could also have been employed in this study for quantitative assessment of MHC expression.

Once the qualitative study of surface MHC antigen expression on the HCC cell lines was performed, the quantitative assessment of antigens was performed using the ELISA. This assay also detects surface antigens but at a higher level of sensitivity than IIF (to date, there is no literature that records the level of sensitivity for this type of ELISA). Through a series of dose response experiments, the dilutions of the monoclonal and enzyme-labelled antibodies that resulted in maximal detection of the surface antigens were obtained. These optimal dosages were used for all further ELISAs. The ELISA detected MHC spontaneous expression of Class I surface antigens on all the HCC cell lines investigated. MHC Class II antigen display was observed under spontaneous condition on three HCC cell lines, PLC/PRF/5, HA22T/VGH and HA59T/VGH. The ELISA also confirmed the study's assignment of a negative control to the K562 cell line and positive to Raji. This assay is again, rapid and relatively cheap (Watt et al 1988).

The optimal quantitative assessment of MHC surface expression is flow cytometry. This assay is highly sensitive but requires a specially trained operator and expensive equipment. Krief et al (1988) determined HLA expression on several HCC cell lines (HA22T/VGH, PLC/PRF/5, Li7A, HCC36, Hep 3B and HEP G2) using flow cytometric

analysis. The finding by Krief et al (1988) that PLC/PRF/5 does not spontaneously express MHC Class II antigens is in contrast to that of the present study. A possible explanation for this anomalous result is that different generations of the cell line was used in the present study and that of Krief et al (1988). It is not unreasonable to suggest that the expression of these surface antigens vary from one generation to the next (Freshney 1987). Thus some workers may have available a generation of PLC/PRF/5 that does express low amounts of MHC Class II antigens (Franco et al 1988) while others do not (Krief et al 1988).

The present study investigated the effect of the modulating agents r-IFN-alpha and -gamma on MHC expression on the HA22T/VGH, TONG PHC and HA59T/VGH cell lines. MHC class I expression was enhanced on TONG PHC by 200 IU/ml r-IFN-alpha after a 24 hour incubation period. This finding of MHC class I enhancement by r-IFN-alpha is in agreement with that observed by Krief et al (1988).

These workers also observed that in HCC cell lines an inverse relationship existed between the possible enhancement of class II antigens by r-IFN-gamma and the degree of cellular differentiation. They found that

10 IU/ml of glycosylated IFN-gamma is sufficient to dramatically enhance MHC class II expression on HA22T/VGH. However Franco et al (1988), investigating the effect of IFN-gamma on the well-differentiated cell line PLC/PRF/5, found that this modulating agent caused the enhancement of MHC class II antigen expression. In this present study, the moderately differentiated cell line, TONG PHC (Lin et al 1984), displayed a marked enhancement of MHC class II expression after incubation with 200 IU/ml of r-IFN-gamma for 24 hours. The poorly differentiated cell line, HA59T/VGH, displayed enhanced MHC class II expression but this was not as marked as that observed in TONG PHC. This study, therefore, did not confirm the inverse relationship observed by Krief et al (1988).

The chemical sodium butyrate enhanced the expression of MHC on all cell lines investigated: TONG PHC, HA22T/VGH and HA59T/VGH. This effect was most marked on TONG PHC, moderate on HA22T/VGH and in the cell line HA59T/VGH the effect of sodium butyrate on MHC expression was minimal. In TONG PHC and HA22T/VGH, the effect of sodium butyrate on MHC expression was dose dependant with the optimal dose occurring in the range of 2 mMolar. The effect of this

chemical was most marked on MHC class II expression. Optimal incubation time for sodium butyrate to obtain maximal differentiation between stimulated and resting cells was 24 hours.

Clofazamine had a variable effect on MHC Class I expression. Incubation of cells with this chemical, however, resulted in an enhancement of MHC class II expression on HA22T/VGH, HA59T/VGH and TONG PHC. Effect of clofazimine on MHC class II expression was most marked on TONG PHC and HA59T/VGH and least marked on HA22T/VGH.

It is difficult to infer MHC gene expression from studies using protein analysis techniques (eg Western blotting), as it is possible that mRNA becomes degraded by cellular ribonucleases before translation occurs (Sambrook et al 1989). The most definitive method for establishing whether or not MHC gene products are being expressed, is therefore to examine the genes themselves. This may be achieved by dot-blot or Northern Blotting (mRNA) analysis which establishes the pre-or post-translational status of the genes. Di Bisceglie et al (1986) employed mRNA Northern blotting to determine AFP status in HCC in vivo and the PLC/PRF/5 cell line. There are no accounts of this technique being adopted by other workers in examining the

status of MHC antigens on HCC cell lines. All studies have concentrated mainly on surface or cytoplasmic expression (Krief et al 1988). Thus the examination of MHC expression at a gene level should be further investigated.

CHAPTER 6 POSSIBLE FUTURE INVESTIGATIONS

6.1 INTRODUCTION

Analysis of the DNA coding for the major histocompatibility gene products elucidated the complexity as well as the complete amino acid sequence of the glycoproteins (Lee et al 1982). The general approach to obtaining complementary DNA (cDNA) clones is to firstly obtain RNA enriched with the mRNA encoding for a particular gene from a homozygous cell line eg. Raji (Claesson et al 1983). Double stranded cDNA is synthesised from the resulting enriched mRNA using reverse transcriptase and then inserted into the appropriate restriction site of a suitable plasmid. Subsequent clones are derived from transformation experiments with the plasmid and the DNA sequence of the clones then analysed and the recombinant products characterised. Such as cDNA clones coding or the heavy chain of HLA-DR antigens Since antisera against the Ii chain were not available at the time, the apparent molecular weight was used as the characteristic of the gamma chain in the screening of the clones (Claesson et al 1983). In their analysis of sequencing of the isolated cDNA clones, Claesson and colleagues determined that the invariant chain was inverted when compared with the HLA-DR antigen, ie. the NH_2 terminus of the Ii chain resided on the cytoplasmic side of the membrane (Claesson et al 1983). The reasons for this are not yet known precisely but it has been suggested that the complexity of HLA Class I antigens

serves an important function (Ploegh et al 1981). Establishment of cDNA clones not only allows for the protein sequence and characterisation of the antigen for which the clone encodes (Trowsdale et al 1984) but also permits the study of the expression of mRNA HLA products using Northern blot hybridization (Kawata et al 1984).

This dissertation has investigated the expression of MHC antigens on certain hepatoma derived cell lines. Methods of detection used in this study have quantitatively as well as qualitatively examined surface expression of these antigens. In addition, MHC expression on three cell lines has been studied after the addition of certain lymphokines and chemicals. However, it is apparent that further investigations examining the MHC gene expression as well as the pre- or post- translational status of these gene products are required. Some of these studies have already been initiated. These investigations are:

a) The isolation of total cytoplasmic RNA from the various cell lines and

b) The purification, isolation and characterisation of cDNA probes directed against the MHC (messenger RNA).

The findings presented in Chapter 6 have demonstrated that isolation of intact cytoplasmic RNA from various HCC and

control cell lines using a guanidium thiocyanate method is possible. The isolation and subsequent purification of cDNA probes has also been demonstrated.

Further studies that need to be undertaken to investigate the pre- or post-translational status of the MHC gene products are:-

a) The study of the expression of HLA mRNA using Northern blot hybridization.

b) The effect that the addition of lymphokines and chemicals has on the amount of MHC mRNA produced as detected by Northern blotting. That is, can the addition of a particular lymphokine cause an increase or decrease of MHC mRNA isolated from a cell line.

The above studies would allow the comparison of the findings obtained using the ELISA and the IIF technique and those obtained from molecular biological techniques.

6.2. MATERIALS AND METHODS

6.2.1. Preparation of competent E.coli JM101 cells

A culture of E.coli JM101 cells (kind gift of V Mizrahi, SAIMR, Jhb, SA) was grown up overnight at 37°C with shaking in 5ml minimal medium (Appendix 5). Thereafter, 50 ml LB medium was innoculated with 0,5ml of the overnight culture and the culture grown at 37°C with

vigorous aeration (Appendix 4) . Cells were grown until an OD at 650nm of 0.3-0.4, stored on ice for 15 minutes and then harvested by centrifugation for 10 minutes at 4000g at 4°C in a pre-cooled JA20 rotor (Beckman Instruments, Palo Alto CA, USA). After resuspension in 10ml (1/5 original volume) ice-cold 0,1M $MgCl_2$, the cells were immediately centrifuged for 15 minutes at 2000g at 4°C in the JA20 rotor. Cells pelleted as a ring at the bottom of the tube and were gently resuspended in 10ml (1/5 original volume) ice-cold 0,1M $CaCl_2$. After cells had been stored on ice for 60 minutes, they were centrifuged for 15 min at 4°C at 2000g in the JA20 rotor. Once again, cells pelleted as a distinct ring at the bottom of the tube and were resuspended in 2ml (1/25 original volume) of ice cold 0,1M $CaCl_2$. Competent cells were either used immediately or stored on ice for up to 24 hours, after which they had lost viability and were unsuitable.

6.2.2 TRANSFORMATION OF E.COLI JM101 CELLS

For each transformation, 0,2ml competent E.coli JM101 cells were used. Depending on the concentration of DNA (Appendix 5), approximately 75 ul (equivalent to 2ng DNA) of the DNA- containing solution suspended in Tris-EDTA (TE) (Appendix 4) was used for each transformation. The following cDNA probes were used in this study: 8-ABC-5 (anti-HLA-A,B,C), MCFI-1B (anti-HLA-DRß) and p-gamma-2

TABLE 6.1 DESCRIPTION OF cDNA PROBES USED IN THIS STUDY

cDNA probe	Directed against	Plasmid	Insert (bp)	Restriction Site	Reference
8-ABC-5	HLA-A,B,C	pAT 153	669	Pst I	Trowsdale et al (1984)
MCFI-1ß	HLA-DRß	pBR322	510	Pst I	Kind gift of J Trowsdale (ICRF,London, U.K.)
p-gamma-2	Ii	pBR322	1287	Pst I	Claesson et al (1983)

The 8-ABC-5 and p-gamma-2 recombinant plasmid were conferred with tetracycline resistance while MCFI-1ß was ampicillin resistant.

(anti-Ii). A full description of these probes is given Table 6.2. After addition of the DNA-containing solution, and gentle pipetting to ensure mixing, the cells were stored on ice for 60 min. in 1,5 ml Eppendorf tubes (Eppendorf, Germany). A positive control of 2ul Gal KRT' (1ng/ul) having ampicillin-resistance (amp^r) was used in this study. After heat-shocking at 42°C for 2,5 minutes, 7 ml LB medium (Appendix 5) was added with gentle mixing and the cells incubated for 60 min at 37°C. Different volumes (25, 50, 300, 700 ul) of the transformed E.coli JM101 cells were then spread on pre-warmed sterile agar plates (Appendix 5) innoculated with appropriate antibiotic resistance. Plates having amp^r were incubated at 37°C for not longer than 18 hours while those have tet^r were incubated in the dark at 37°C for up to 36 hours. If colonies were observed on the amp^r plates on which Gal KRTE had been spread, it was assumed that the transformation was successful.

6.2.3. ISOLATION AND PURIFICATION OF cDNA PROBES

6.2.3.1 Initial isolation of cDNA probes using a rapid plasmid DNA minipreparation

The protocol, as outlined by Sambrook et al (1989), was followed for the rapid plasmid minipreparation of the isolation of cDNA probes. Briefly, a culture of the transformed E.coli JM101 cells was incubated overnight

at 37°C with shaking in 15 ml polypropylene round bottomed tubes (Falcon, Becton Dickinson, NJ, USA) in 1,5 ml LB medium (Appendix 5) containing appropriate antibiotics (10 ug/ml ampicillin, 50 ug/ml tetracycline). The minipreparation was carried out on six colonies picked from the same agar plate which was incubated with a plasmidcontaining strain. Approximately 150 ul of the overnight culture was kept for further plasmid purification experiments and the cells of the remaining culture harvested by centrifugation for 5 min at 1200g at 4°C in an Eppendorf microfuge. The supernatant was then gently aspirated and the cells resuspended in 100 ul ice-cold lysis buffer solution I (Appendix 5) by thorough pipetting. After transfer to sterile 1,5 ml Eppendorf tubes (Eppendorf, Germany), incubation at room temperature for 5 minutes followed and then 200 ul of room temperature solution II (Appendix 5) was added to the cells. The suspension was carefully mixed by inversion of the closed tube. The solution turned viscous and partially clarified and was incubated on ice for 5 minutes. The addition of 50 ul ice-cold Solution III (Appendix 5) and mixing by vigorous inversion resulted in a thick, white precipitate. The suspension was stored on ice for 5 min and thereafter centrifuged for 5 minutes at 4°C in an Eppendorf microfuge. Approximately 400 ul of the supernatant was carefully removed, without disturbing the fluffy white

pellet, and transferred to a tube containing 400 ul PhOH/$CHCl_3$ 1:1 (Phenol was equilibrated to a pH of 8.0 with Tris HCl (pH 8.0) according to methods outlined by Sambrook et al (1989)). After vigorous vortexing and centrifugation for 5 minutes at 4°C in an Eppendorf microfuge, the aqueous phase was transferred to another sterile Eppendorf tube containing 400 ul PhOH/$CHCl_3$ 1:1 and the extraction repeated. The top phase (approximately 400 ul) was transferred to a tube containing 200 ul $CHCl_3$, vortexed and the suspension centrifuged at room temperature for 2 min in an Eppendorf microfuge. After transferral of the top (aqueous) phase (approximately 400 ul) to a fresh tube, 1 ml ice-cold 95% EtOH was added and the solutions mixed by inversion. DNA and RNA was precipitated by centrifugation for 10 minutes at 4°C in an Eppendorf microfuge. The supernatant was poured off and the pellet washed with 1 ml ice-cold 70% EtOH. After centrifugation for 5 min at 4°C in an Eppendorf microfuge and careful aspiration of the supernatant, the pellet was dried in a vacuum centrifuge (SpeedVac Concentrator, Savant, Hicksville, NJ, USA) for 5 min. Thereafter, the DNA-RNA pellet was dissolved in 25 ul TE/RNase A (Appendix 5) and the RNA destroyed by heat-inactivation at 60°C for 10 min. After thorough mixing and recentrifugation at room temperature for 1 min in an Eppendorf microfuge the DNA solutions were stored at -20°C until needed.

Plasmid DNA was digested with appropriate restriction enzymes (Table 6.1) and the entire digested sample run on a 1,5% agarose minigel (Hoeffer Scientific Instruments, San Francisco, CA, USA). A series of digestions with different restriction enzymes (Bam HI, Hinc III, Pst I) were used until the correct insert as assessed by molecular weight markers (Boehringer Mannheim, Mannheim, Germany) was found and this plasmid DNA minipreparation was used for further purification.

6.2.3.2 PLASMID PURIFICATION

Approximately five hundred microlitres of an overnight culture of E.coli containing plasmids (Table 6.1) was grown at 37°C in 5ml LB medium (Appendix 5) supplemented with appropriate antibiotics (Table 6.1). The culture was innoculated into 500 ml LB medium (Appendix 5) supplemented with appropriate antibiotics (Table 6.1) (if plasmids exhibited tet^r, cultures were kept in the dark) and grown with vigorous shaking (300 rpm) until the optical density (OD) at 600 nm was 0.5. When this OD was reached (ie cells were in midlog phase), 2,4 ml of a chloramphenicol (34 mg/ml) solution was added to amplify copy number of the plasmid while inhibiting bacterial replication. The culture was grown for not longer than 16 hours at 37°C with shaking (120 rpm). Cells were pelleted for 15 min at 3000g at 4°C in the JA20 rotor and resuspended in 4 ml solution I (Appendix 5). The solution was transferred to

a clean tube and 1 ml of a freshly made up lysosome (50 mg/ml) solution added (Boehringer Mannheim, Mannheim, Germany). After gentle swirling, the suspension was incubated at room temperature for 5 min. Ten ml of solution II (Appendix 5) was added, the tube gently inverted and then incubated on ice for 10 min. After addition of 7,5 ml solution III (Appendix 5), the tube was inverted sharply four times and the suspension incubated on ice for 10 minutes. The resulting white precipitate was centrifuged at 10000g for 15 min and the supernatant transferred to a clean tube. Twelve ml of room temperature isopropyl alcohol was added, the suspension gently mixed and stored at room temperature for 15 min. Centrifugation at room temperature at 10000g for 15 min recovered the nucleic acid pellet and the supernatant was discarded. The pellet was vacuum dried and resuspended completely in exactly 4,2 ml TE (Appendix 3) and transferred to a polyallomer quick-seal centrifuge tube (Beckman, Palo Alto, CA, USA (13 x 51 mm). To the solution was added 4,8g CsCl (Boehringer Mannheim, Mannheim, Germany) and 0,42 ml ethidium bromide (10 mg/ ml). Thereafter the tube was kept in the dark. The tubes were sealed after weighing (Tube topper for quick seal tubes, Beckman Instruments) and spun in a Beckman VTI 50 vertical rotor at 150000g for 18 hours at 15^{o}C. After centrifugation, the upper band containing plasmid DNA was removed using a 23-gauge syringe needle

attached to a syringe and disposed into a 1,5 ml eppendorf tube. An equal volume of butanol (equilibrated with 20 x SSC (Appendix 6) in a ratio of 1:1) was added to remove ethidium bromide. Extraction of the dye was repeated until the upper phase was completely colourless. The lower phase containing plasmid DNA was transferred to polycarbonate ultraclear centrifuge tubes (Beckman), two volumes TE and 2,5 volumes 95% EtOH were added and the DNA pellet collected by centrifugation for 15 min at 15000g at room temperature in the JA20 rotor. The pellet was vacuum-dried, resuspended in 500 ul TE buffer (Appendix 4) and the OD at 260 nm and 280 nm measured to determine the DNA concentration and its purity.

6.2.3.3 ELECTROELUTION OF ISOLATED cDNA PROBES

Electroelution was carried out using a level unidirectional electroeluter (International Biotechnologies Inc, New Haven, Conneticut, USA) using a low salt electroelution buffer (Appendix 5). After excision of the agarose containing the correct insert from a 1,5% agarose gel at 75 mV and 120mA for 120 min (Minnie submarine agarose unit, Hoefer Scientific Instruments, San Francisco, CA, USA) thegel slice was loaded onto a trough containing 100 ul high salt buffer (Appendix 6) DNA was electroeluted (100V, 50mA) for 30 minutes and 400 ul withdrawn from the trough. Ethidium bromide and the loading dye were extracted three times using 300 ul saturated butanol

(butanol:20 x SSC:1) (Appendix 6) for every extraction procedure. DNA was precipitated by addition of 1ml ice-cold 95% EtOH and incubation for 60 min at -20°C. After centrifugation at 4°C for 30 min, the supernatant discarded and the DNA pellet vacuum dried (SpeedVac Concentrator, Salant, Hicksville, NJ USA). The purified cDNA was dissolved in 50 ul TE (Appendix 4) and stored at -20°C until needed.

6.2.3.4 PREPARATION OF RNA FROM HCC, RAJI AND K562 CELL LINES

Total RNA was isolated by the guanidine thiocyanate methods of Chirgwin et al (1979). Approximately $5x10^6$ cell/ml were harvested according to cell culture methods outlined in Section 2.4.3 and washed twice with PBS (Appendix 1). The cell pellet was resuspended in 3.2 ml of 4M guanidine thiocyanate solution (Appendix 5) and the suspension vortexed for 20 seconds. The lysate was carefully layered onto 1.2 ml of a 5.7M CsCl solution (Appendix 6) in a 5 ml centrifuge tube (Beckman Instruments Inc, Palo Alto, CA, USA) and centrifuged for 18 hours at 110000g at 20°C in a SW55 rotor (Beckman). The supernatant was carefully removed and the RNA pellet resuspended in a total of 400 ul of diethyl pyrocarbonate (DEPC) treated water (Appendix 5). The RNA solution was transferred to a 1.5 ml eppendorf tube (Eppendorf, Germany) and 40 ul of 3 M sodium acetate

(pH 6) added to the RNA mixture followed by 900 ul 95% ethanol. The suspension was thoroughly mixed by vortexing. After precipitation at -20°C overnight, the nucleic acid suspension was recovered by centrifugation in an Eppendorf microfuge for 30 min at 4°C. The supernatant was discarded and the RNA pellet dried in a vacuum centrifuge (Savant Instruments, Hicksville, NY, USA). The pellet was resuspended in 50 ul of DEPC-treated water, the RNA concentration determined by OD at 260 nm and the RNA solution stored at -20°C until needed.

6.2.3.5 ELECTROPHORETIC SEPARATION OF RNA.

Total RNA extracted by the guanidine thiocyanate method was resolved by agarose gel electrophoresis in the presence of formaldehyde according to the method of Lehrach et al (1977). Two grams of agarose (Bio-Rad Laboratories, Richmond, CA, USA) was dissolved by heating in 160 ml distilled water and cooled to 50°C before addition of 20 ml of 10 x MOPS buffer (Appendix 6) and 20 ml of 37% formaldehyde. A horizontal minigel electrophoresis apparatus was used (Hoefer Scientific Instruments, San Francisco, CA, USA). RNA was denatured in 9-12 ul of loading buffer (Appendix 6) by heating to 56°C for 15 min. Equal amounts of each RNA sample (4 ug) were applied to wells in 5 mm thick agarose formaldehyde gels. Electrophoretic resolution was carried out using 1 x MOPS as the running buffer at 4°C for 18-20 hr at 35-40 V per gel.

6.2.3.6 PRELIMINARY FINDINGS

Preparation of competent E.Coli JM101 cells using the alkaline lysis method and their subsequent transformation was successfully performed. Initial isolation of the cDNA probes 8-ABC-5 (anti-HLA-A,B,C), MCFI-1B (anti-HLA-DRB) and p-gamma-2 (anti-Ti) using a rapid plasmid DNA minipreparation was achieved. Figure 6.2 shows the restriction fragments of the cDNA 8-ABC-5 that was obtained after subsequent digestion with the appropriate restriction enzyme, Pst I. In Figure 6.2, the probe 8-ABC-5 (anti-HLA-A,B,C) is visualised with ethidium bromide using agarose gel electrophoresis. The insert of 693 bp is also shown. The plasmid and its insert were of reasonable purity. According to the restriction map shown in Fig 6.1a, the cDNA probe 8-ABC-5 is 669bp long.

After it was ascertained that the plasmid containing the correct insert had been isolated, the three plasmids were purified on a larger scale using a CsCl gradient. The plasmid was again digested with the appropriate restriction enzymes and the correct insert further purified by electroelution. Figure 6.3 shows the restriction fragments obtained after digestion of the plasmids from the large scale plasmid preparation. In this figure, the cDNA probes

FIGURE 6.1 RESTRICTION MAPS.

FIGURE 6.1a

Schematic map of recombinant plasmid pHLA-A containing 669 bp long insert of the cDNA probe 8-ABC-5. The numbers refer to base-pairs (Redrawn from Trowsdale et al 1984).

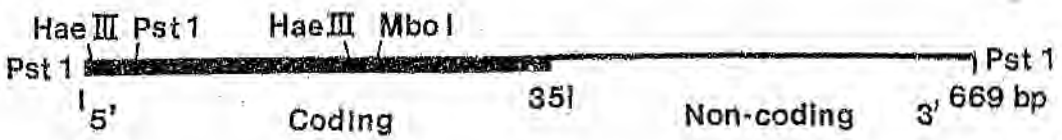
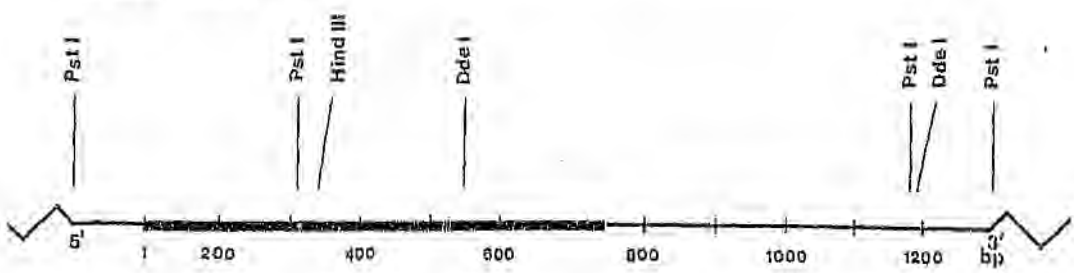


FIGURE 6.1b

Restriction map of the p-gamma-2 cDNA probe containing the Pst I 1287bp insert. The 5' end of the coding strand is close to the ECORI site in pBR322. The thick line represents the translated portion. (Redrawn from Claesson et al 1983).



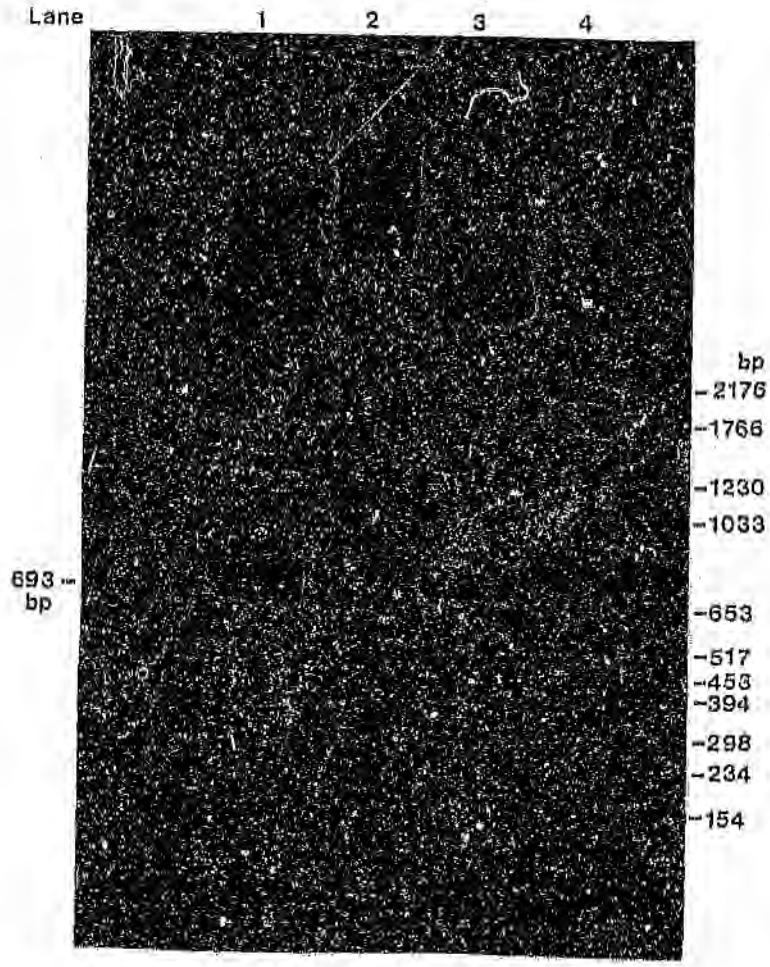


FIGURE 6.2

Example of rapid plasmid preparation of recombinant plasmid pHLA-A (lane 2). The Pst I insert of 695bp is shown in lanes 1 and 4. The molecular weight standards are shown in lane 8.

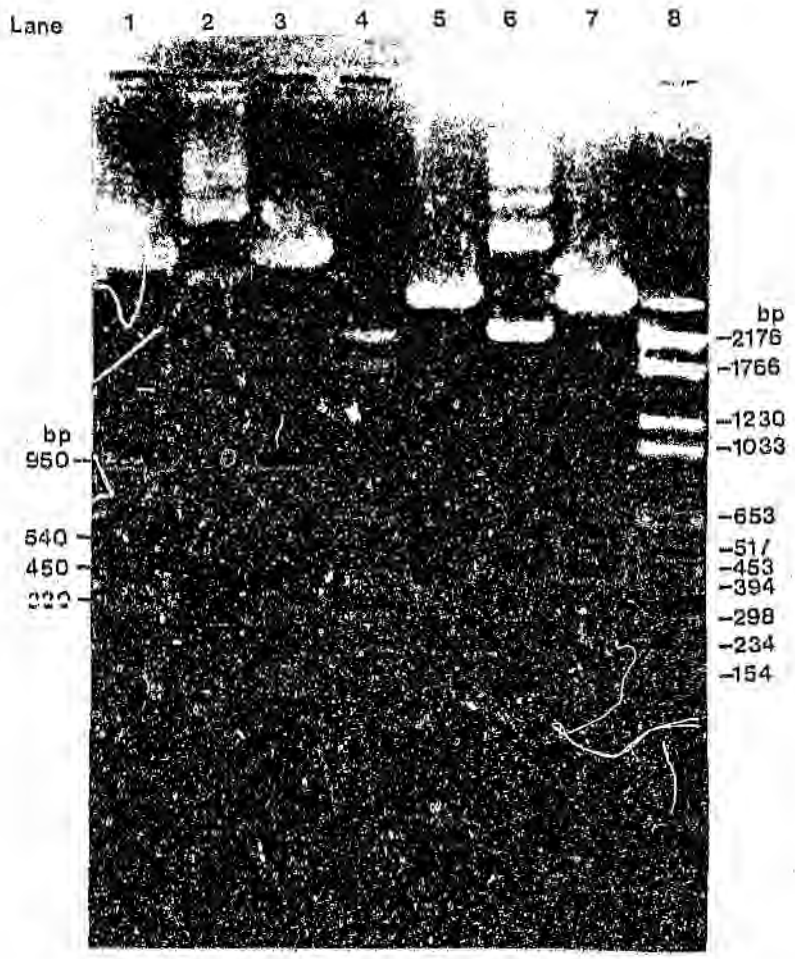
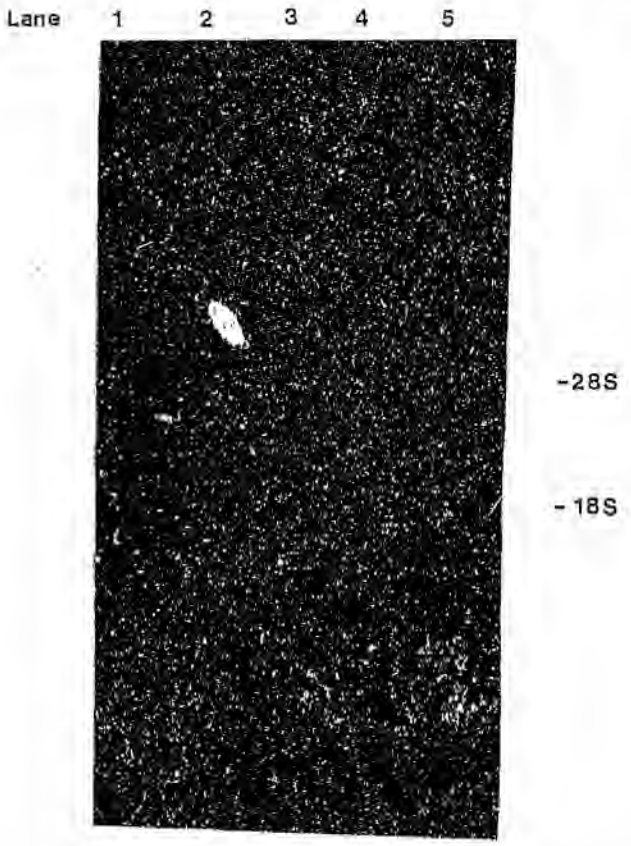


FIGURE 6.3

Example of plasmid preparation of recombinant plasmid p-gamma-2 (lane 2) showing two Pst I inserts (lanes 1 and 3) of 950 and 330 bp each. The plasmid preparation of recombinant plasmid MCFI-1ß (lane 6) shows two Pst I inserts (lanes 5 and 7) of 540 and 450 bp each. The molecular weight standards are shown in lanes 4 and 8.

p-gamma-2 and MCFI-1ß which were successfully isolated were visualised using ethidium bromide. Their size in bp was assessed using molecular weight standards. In this figure, two restriction fragments of 950 and 330 bp each were obtained after Pst I digestion. It is possible to use both restriction fragments for further experiments such as in Northern blotting. This is suggested by the restriction map of p-gamma-2 which indicates the 1287 bp insert containing four different Pst I sites (Figure 6.1.b). Also shown in Figure 6.3 is the plasmid preparation of the recombinant plasmid MCFI-1ß. Two Pst I digests of 450 and 540 bp each were obtained. It is possible to use both fragments for further cDNA probing according to the restriction map. Although unpublished, this was given via a personal communication of Dr J Lee (ICRF, London, UK).

Total cytoplasmic RNA was prepared from the Raji, K562 and the HCC cell lines that were examined in this study. Formaldehyde gel electrophoresis separated the intact RNA into the 18S and 28S rRNA species. As shown in Figure 6.4, the 18S and 28S rRNA species of the HA59T/VGH, HA22T/VGH, TONG PHC, PLC/PRF/5 and Hep 3B were visualised under fluorescence using the intercalating agent, ethidium bromide.



Lane 1: HA59T/VGH
2: HA22T/VGH
3: Tong PHC
4: PLC/PRF/5
5: Hep 3B

FIGURE 6.4

Example of formaldehyde agarose gel separation of RNA isolated from five different HCC cell lines (lanes 1-5) showing 18S and 28S rRNA species.

6.3 DISCUSSION

The isolation and purification of cDNA probes was achieved by alkaline lysis according to methods outlined by Sambrook et al (1989). This method allows for quick and clean minipreparations of plasmid DNA. Figure 4.2 shows the restriction fragment of approximately 693bp obtained after Pst I digestion of a representative cDNA probe, 8-ABC-5. The Pst I insert is well separated and of a reasonable purity. Although the running of a CsCl gradient is long and expensive (18 hours at 150 000g). The large scale plasmid preparation results in clean, and well-resolved plasmids. These plasmids were of a good purity and free of protein contaminant (Figure 6.3). In Figure 6.3, both the probes MCFI-1ß (550 bp insert) and p-gamma-2 (1280 bp insert) where digested by Pst I. Each probe was resolved into two well-separated restriction fragments by agarose gel electrophoresis. Visualization using ethidium bromide showed that MCFI-1ß (anti-HLA-DRß) was digested into two Pst I fragments of approximately 540 and 450 bp each (as assesed by molecular weight standards). This is possible according restriction map of this cDNA probe obtained via a personal communication of Dr J Lee, ICRF, London, UK. The two digests obtained from the Pst I restriction of p-gamma-2 were confirmed by referral to the restriction map of this cDNA probe (Fig 6.1b). This map details the Pst I restriction sites which would give rise to three fragments

of an estimated 940, 330 and 120 bp each. Subtraction of the 95 bp initiation codon described by Claesson et al (1983) results in a Pst I insert of approximately 1295 bp. Figure 6.3 showed two Pst I digests of 950 and 330 bp each. The smaller restriction fragment of approximately 120 bp was not visible. There are two possible reasons for this. Firstly, because of its small number of base pairs, this fragment was not resolved in the 1,5% agarose gel used in this study (that is, a higher concentration of agarose was needed to resolve this band) or secondly, the concentration of DNA loaded in the well was too weak for this fragment to be observed at the front of the band. The absence of this band does not affect the probing ability of p-gamma-2 since both the visible fragments are suitable for Northern blotting.

Figure 6.4 shows a representative formaldehyde agarose gel separation of total RNA isolated from five HCC cell lines. The resolution of RNA into 18S and 28S rRNA species implied that RNA was intact ie undegraded. Degraded RNA, which appears as a smear on the formaldehyde agarose gel is unsuitable for Northern blotting and hybridization experiments. It is essential to extract RNA under sterile conditions. The lability of the 3'-hydroxyl group on uracil easily causes degradation of the RNA sample by hydrolysis. For this reason, all methods and reagents

which inhibit ribonuclease activity are favoured when extracting RNA from cells with high activity of this enzyme (Sambrook et al 1989). In this study, the method of Chirgwin et al (1979) was employed for the preparation of biologically functional RNA. This method employs two powerful RNase inhibitors: diethylpyrocarbonate and guanidium thiocyanate. It was initially determined that RNase had a half-life of 10 sec in 4M guanidium hydrochloride. However, later it was demonstrated that guanidium thiocyanate has a much more rapid denaturant of RNase permitting the isolation of intact RNA (Chirgwin et al 1979) as demonstrated by the presence of intact 28S and 18S rRNA species (Fig 6.4). Total RNA preparations had an OD 260/OD 280 ratio of between 1,8 and 2,2 indicating low protein contamination. The use of a CsCl gradient improved the purity of the RNA preparation. RNA prepared with guanidium thiocyanate was shown to retain the information content of the starting RNA and was fully active in translation, hybridization and recombinant DNA experiments (Chirgwin et al 1979). Thus this method of extracting biologically active RNA is suitable for further hybridization experiments with cDNA probes. Northern blotting and subsequent hybridization experiments using the cDNA probes purified and the RNA extracted from the HCC cell lines will be performed at a later date.

APPENDIX 1

MATERIALS FOR ENZYME-LINKED IMMUNOASSAY ELISA

Sodium Carbonate Buffer (coating buffer) 0.05M

	1 Litre
$NaCO_2$ (BDH, Poole, England)	1.590g
$NaHCO_3$ (BDH, Poole, England)	2.930g

Make up to 900ml with distilled H_2O

Adjust pH to 9.6

Make up to 1 litre with distilled H_2O

Store in 100 ml aliquots at -20°C.

0.5% Bovine Serum Albumin (BSA)

Dissolve 0.5g BSA (Seravac, Pentax Products, Illinois, USA) in 100ml sodium carbonate buffer

Adjust to pH 9.6.

Store at -20°C.

Phosphate Buffered Saline (PBS)

0.15M (10x concentration)	1 litre
NaCl (Univar, South Africa)	80g
KCl (Univar, South Africa)	2g
$Na_2HPO_4.2H_2O$ (BDH, Poole, England)	11.5g
KH_2PO_4 (BDH, Poole, England)	2g

Dissolve in 900ml distilled H_2O.

Adjust the pH to 7.2.

Make up to 1 litre with distilled H_2O.

Sterilise by autoclaving.

PBS-Tween 0.05%

Add 0.5 ml Tween-20 (polyoxyethelene sorbitan monolaurate, Sigma Chemical Co., St Louis, Missouri. USA) to 1 litre PBS.

APPENDIX 2

PEROXIDASE SUBSTRATE FOR ELISA

Substrate Buffer

Citric acid (BDH, Poole, England)	1.029g/100ml
Na_2HPO_4 (BDH, Poole, England)	1.448g/100ml

Dissolve in distilled H_2O

Adjust to pH 5.0

Store in the dark

Make up fresh daily

Substrate

Immediately prior to use add [illegible] 0% H_2O_2 (BDH Chemicals Ltd., Poole, England) and 34mg O-phenylenediamine (Sigma Chem. Co., St Louis, MO, USA) to 100ml substrate buffer.

Keep the substrate solution entirely covered until used.

APPENDIX 3

CELL CULTURE PROCEDURES

Preparation of RPMI-1640 medium[a]

Per litre:

RPMI-1640 (10x)	100 ml
L-Glutamine	2 mM
$NaHCO_3$ (7,5%)	2200 mg/l
Hepes (1M)	20 mM
FCS (heat inactivated)[b]	100 ml[c]
Gentamycin	0,1%
Penicillin, Streptomycin, Fungizone (100x)	1%
Sterile, autoclaved H_2O	900 ml

Add ingredients together[d] and sterilise by autoclaving. Store complete medium (RPMI-1640 supplemented with FCS) at 4°C until needed.

Preparation of DMEM medium[a].

per litre:

DMEM (5x)	200 ml
$NaHCO_3$ (7,5%)	3700 mg/l
Hepes (1M)	20 mM
FCS (heat-inactivated)[b]	100 ml
Gentamycin (1%)	0,1%
Penicillin, Streptomycin, Fungizone (100x)	1%
L-Glutamine	4 mM
Sterile autoclaved H_2O	800 ml

Add ingredients together[d] and sterilise by autoclaving. Store complete DMEM (DMEM supplemented with FCS) at 4°C until needed.

a) all media ingredients were obtained sterile from Highveld Biologicals, Johannesburg, SA.
b) Fetal calf serum (FCS) was heat-inactivated for 30 min at 56°C and stored at -20°C in 50 ml aliquots. Cells grown in suspension were grown in RPMI-1640 medium supplemented with 5% FCS (50 ml/l). HCC cells were grown in DMEM supplemented with 10% FCS (100 ml/l).
c) Medium was used at a pH of between 7,2 and 7,4 and was adjusted with 1N NaOH.

Procedure for the preservation of cells

Trypsination of cells

Cells were preserved by freezing in liquid nitrogen in the presence of the cryoprotective agent, glycerol.

Glycerol	10%
FCS	40%
Liquid medium	50%

Glycerol was sterilised by autoclaving and added to FCS and liquid medium. The mixture was filter-sterilised with a 0,22 um filter (Millipore, Bedford, MA, USA) and used immediately. Cells that had been counted and their

viability assessed were suspended in 2 ml glycerol freezing medium and the mixture transferred to plastic ampoules (Cel-Cult, Hounslow, England). Ampoules were stored in liquid nitrogen.

Procedure for the thawing of frozen cells

For reconstitution of frozen cells, ampoules were removed from liquid nitrogen and immediately transferred to a 37°C waterbath. Cells were harvested by centrifugation at 200 g for 5 min after careful addition of 10 ml complete medium at 37°C. Viability was assessed and the cells plated out on cell culture flasks.

APPENDIX 4

STOCK SOLUTIONS USED FOR ISOLATION OF cDNA PROBES

0,5M EDTA (pH 80)

Disodium ethylenediamine	
tetraacetate.2 H_2O	93,05 g
deionised H_2O	800 ml

Stir until dissolved, then adjust pH to 8.0 with NaOH pellets.

Sterilise by autoclaving.

10% Sodium dodecyl sulphate (SDS)

Electrophoresis-grade SDS	10 g
(Fluka Biochemie, Switzerland)	
deionised H_2O	90 ml

Heat until dissolved (70°C) then adjust pH to 7.2 with concentrated HCl. Adjust volume to 100 ml. Sterilise by autoclaving.

Tris-HCl (1M)

Tris (hydroxymethyl) methylamine	
(Tris base)	121,1 g
deionised H_2O	800 ml

Adjust pH to 7,5 and 8.0 with concentrated HCl. Sterilise by autoclaving.

Tris. EDTA (TE)

10 mM Tris.HCl (pH 9.0 1M)

1 mM EDTA (pH 8.0 0,5M)

Ethidium Bromide (10 mg/ml)

Ethidium Bromide (Boehringer Mannheim, Mannheim, Germany)	1 g
H_2O	100 ml

Stir overnight at room temperature.

Wrap container in aluminium foil.

Store at 4°C.

Potassium Acetate (5M)

CH_3COOK (BDH Ltd, Poole, England)	122,68 g
deionised H_2O	220 ml

Adjust pH to 7.5 with acetic acid (2M).

Make up to 250 ml with H_2O.

Sterilise by autoclaving.

Tris-Acetate (TAE)

50x stock solution:

Tris base (pH 8.0 1M)	242 g
Glacial acetic acid	57,1 ml
EDTA (pH 8.0, 0,5 M)	100 ml

Make up volume to 1 litre with deionised H_2O.

Working solution (1xTAE)

0,04 M TAE

0,001 M EDTA

APPENDIX 5

SOLUTIONS USED IN cDNA PREPARATIONS

1) 1xM9 Minimal bacterial growth medium

a) M9 salts (5 x concentrate)

$Na_2HPO4.7H_2O$	30 g
KH_2PO4	15 g
NaCl	2.5 g
NH_4Cl	5 g

Dissolve in deionised H_2O (900ml) and adjust to a final volume of 1 litre with deionised H_2O

Sterilise by autoclaving, and store at 4^oC.

Use 200 ml 5x M9 salt

b) Glucose (20%)

Glucose	20 g
Deionised H_2O	100 ml

Filter sterilise through a 0,45 um filter (Millipore, Bedford, MA, USA). Use 20 ml 20% glucos

Add to 850 ml sterile, deionised H_2O.

(2) Luria-Bertani (LB) Medium

Deionised H_2O	950 ml
Bacto-tryptone	8 g
Bacto-yeast extract	5 g
NaCl	5 g

Adjust pH to 7.4 with NaOH (1M). Bring volume to 1 litre with deionised H_2O and sterilise by autoclaving.

(3) Preparation of Luria Agar (LA) plates

Bacto-tryptone	8 g
Bacto-yeast extract	5 g
NaCl	5 g
Bacto-agar	15 g

Dissolve in 1 litre deionised H_2O
Aliquot into 200 ml portion and sterilise by autoclaving. When agar has cooled to 50^{o}C add antibiotics (50 mg/ml tetracycline-light sensitive, 10 mg/ml ampicillin).

Rapid plasmid DNA preparation

Alkaline lysis method

Solution I

50 mM Glucose (0,5 M)

25 mM Tris-HCl (pH 8.0)

10 mM EDTA (pH 8.0)

Made up to 100 ml with deionised, sterile H_2O.

Solution II

4ml NaOH (10 M)

20 ml SDS (10%)

176 ml H_2O

Make up to 100 ml with sterile, deionised H_2O.

Solution III

60 ml CH_3COOK (5 M)

11,5 ml glacial acetic acid

28,5 ml sterile, deionised H_2O

Preparation of TE/RNaseA

Dissolve pancreatic RNaseA (Boehringer Mannheim, Mannheim, Germany) at a concentration of 2 mg/ml in 10 mM Tris-HCl (pH 7,5 1M) and 15 mM NaCl (5 M). Heat at 100°c for 15 minutes. After cooling until room temperature, aliquot and store at -20°C.

DNase-free RNase A (2 mg/ml)	4 ul
TE	156 ul
Final concentration of RNAseA	50 ug/ml.

Gel-loading buffer (6x)

Bromophenol Blue	0,25%
Xylene cyanol FF	0,25%
Ficoll (Type 400, Pharmacia) in water	15%

Store at room temperature.

Electroelution buffer

High salt buffer

Ammonium acetate (7,5 M)	5,8 g
Bromophenol blue (0,1%)	1 ml

Dissolve salt in 8 ml sterile deionised H_2O and add dye. Adjust final volume to 10ml with sterile, deionised H_2O.

Running buffer (low salt)

Tris HCl (pH 8.0)	20 mM
EDTA	0.2 mM
NaCl	5 mM

Adjust final volume to 1 litre with sterile, deionised H_2O. Sterilise by autoclaving.

APPENDIX 6

RNA PROCEDURES

Diethylpyrocarbonate (DEPC) treated water

Add 1ml DEPC (Fluka Chemie, Switzerland) to 1 litre deionised, sterile H_2O. Mix thoroughly and autoclave. DEPC is light sensitive so bottle is stored in dark.

Guanidine thiocyanate (pH 7.0, 4 M)

Guanidine thiocyanate (4 M) (Merck Chemicals, Darmstadt, Germany)	50 g
Sodium N-lauroylsarcosine (Sarkosyl) (Sigma Chemicals, St Louis MO, USA)	0,5%
2-mercaptoethanol (BDH Chemicals Ltd, Poole, England)	0,1 M
Sodium citrate (pH 7.0, 1 M)	25 mM

After dissolution of solutes in 80 ml DEPC-treated H_2O, filter-sterilise the solution (0,45 um filter, Millipore Bedford, MA, USA) Adjust pH to 7.0 with NaOH (1M), bring final volume to 100 ml and refilter through a 0,45 um filter. Store solution at room temperature in the dark. (GTC is light sensitive).

Cesium Chloride (5.7 M)

Cesium chloride (Boehringer Mannheim Mannheim, Germany)	95,97 g
Sodium acetate (pH 6.0, 3 M)	0,83 ml

Adjust volume to 100 ml with DEPC-treated water. Sterilise by autoclaving.

Morpholinopropane-sulfonic acid (MOPS) buffer (10 x)

MOPS (Boehringer Mannheim, Mannheim Germany)	0,2 M
Sodium acetate (pH 7.0)	0,05 M
EDTA	0,01 M

Loading dye

Formaldehyde (deionised as described in Sambrook et al. 1989)	7,2 ml
10 x MOPS buffer	1,6 ml
Formaldehyde (37%)	2,6 ml
DEPC H_2O	1,8 ml
Bromophenol blue (saturated solution)	0,8 ml
glycerol	1.0 ml

SSC buffer (20x)

NaCl	175,3 g
Na_3 citrate. $2H_2O$	88,2 g
H_2O	800 ml

Adjust pH to 7.0 with HCl (1M). Bring final volume to 1 litre with deionised H_2O. Sterilise by autoclaving.

100x Denhardts

Ficoll 400	10 g
Polyvinylpyrrolidine (PVP)	10 g
BSA (Pentax Fraction V)	10 g

Adjust final volume to 500 ml with sterile deionised H_2O, Filter sterilise (0,45 um Millipore, Bedford MA, USA) and store at -20°C.

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