CLINICAL AND LABORATORY STUDIES IN HEPATOCELLULAR CARCINOMA

A thesis submitted to the Faculty of Medicine,

The University of Glasgow

by

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for the degree of Doctor of Medicine

June 1987

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ACKNOWLEDGEMENTS

This work could not have been completed without the help and support of many people both from within and without the Academic Department of Medicine at the Royal Free Hospital School of Medicine, London, where these studies were undertaken between February 1983 and February 1985.

The technical staff in Professor Howard Thomas' laboratory in the Department of Medicine were infinitely patient in teaching me the laboratory techniques required for my studies. Mr David Brown was particularly helpful and without his aid I doubt that I could have completed my work. Mrs Jaishree Dulabh and the staff of the Comparative Biology Unit were of great help in the studies involving work with animals.

Dame Sheila Sherlock and Professor Neil McIntyre were generous in allowing me to study patients under their care and their constant encouragement was greatly appreciated.

Professor Kenneth Hobbs of the Academic Department of Surgery generously provided resected tumour specimens for HBV-DNA analysis and Professor Peter Scheuer of the Academic Department of Histopathology made light work of reviewing a large amount of histopathological material for me.

Thanks go most of all to Professor Howard Thomas. His perception, infectious enthusiasm for hepatology, approachability and great consideration make him the ideal thesis supervisor. His continued friendship and support is greatly appreciated.

SUMMARY

This work examines a variety of clinical and experimental aspects of hepatocellular carcinoma (HCC), both in patients with this disease and in an animal model of the condition. Most of the studies examine areas of therapeutic interest.

Chapter 1

The clinical, prognostic, aetiological and pathological features of a series of 50 British HCC patients are detailed. Most patients presented in poor clinical condition and with non-specific symptoms. Cirrhosis was present in 78% and approximately 40% of patients had serological evidence of past or present hepatitis B virus infection. Median duration of survival was seven weeks from diagnosis. Only the presence or absence of cirrhosis and clinical condition at diagnosis affected prognosis. The majority of tumours were of trabecular histological pattern and liver cell dysplasia was noted in the non-neoplastic liver of 79% of patients.

Chapters 2 and 3

The treatment of HCC is reviewed and a phase II clinical trial of treatment with the cytotoxic drug mitozantrone reported. Objective responses to mitozantrone treatment were seen in 27% of evaluable patients and serious side-effects were rare. Quality of survival on treatment was good.

Chapter 4

The history of the athymic mouse and the role of this animal in the screening of new anti-cancer therapies is briefly discussed. The "materials and methods" used in the culture of human HCC cell lines and the production of human HCC tumour xenografts are described.

Chapter 5

The effects of human lymphoblastoid interferon (HuIFN-α[Ly], IFN) on the growth of the HCC cell line PLC/PRF/5 were assessed both *in vitro* and in athymic mice with PLC/PRF/5-derived tumour xenografts. *In vitro*, PLC/PRF/5 cells were sensitive to the antiproliferative effects of IFN, growth inhibition being noted at concentrations as low as 1.25 IU/ml, and *in vivo*, IFN reduced tumour xenograft growth rate and prolonged survival in tumour-bearing animals. Further studies examining some of the possible mechanisms involved in growth inhibition *in vivo* demonstrated that IFN was capable of inducing the enzyme 2,5-oligoadenylic acid synthetase, an inhibitor of protein synthesis, in tumour tissue but not in mouse tissue, and that IFN enhanced the display of HLA class I glycoproteins on tumour cells.

Chapter 6

The cytotoxic activity of Natural Killer (NK) cells, which are large granular mononuclear cells of potential importance in the host defence against malignancy, was measured in patients with HCC. Their NK cell activity was found to be reduced when compared to that of healthy control subjects or patients with cirrhosis alone. IFN, at low concentrations, was capable of augmenting the NK cell activity of HCC patients when administered both *in vitro* and *in vivo*.

Chapter 7

Specimens of normal adult human liver and tumour xenografts derived from three human HCC cell lines were examined for the presence of nuclear androgen receptors. Receptors were detected in both benign and malignant tissue, and at similar concentrations ranging from 235-550 fMol mg^{-1} DNA. Castration did not alter significantly the subsequent development and growth of HCC tumour xenografts in male athymic mice inoculated with cells from the PLC/PRF/5 cell line.

Chapter 8

A monoclonal antibody, K-PLC_1, previously produced at the Royal Free Hospital and reacting with a tumour-associated antigen on the cell membrane of PLC/PRF/5 cells, was examined for its ability to bind to HCC cell lines, HCC tumour xenografts, and tumour biopsy specimens from patients with HCC. Using an indirect immunofluorescence technique, K-PLC_1 produced membrane staining of three HCC cell lines and positively stained 10 of 11 human HCC biopsy specimens. In vitro, ^{125}I-labelled K-PLC_1 bound specifically to PLC/PRF/5 cells, and antibody binding could be increased by prior incubation of PLC/PRF/5 cells with high concentrations of human lymphoblastoid interferon. Athymic mice with PLC/PRF/5-derived tumour xenografts were injected with ^{125}I-labelled K-PLC_1 or ^{125}I-mouse IgG, sacrificed at various time intervals, and bound radioactivity then counted in a variety of organs. Though circulating blood-pool radioactivity was high throughout the study period, the amount of ^{125}I-K-PLC_1 was greater in the tumour than in any other solid organ studied and tumour:liver ratios for K-PLC_1 were greater than those for mouse IgG at all time points studied.

Chapter 9

In the light of the results of the thesis, recommendations for further studies are made.

ABBREVIATIONS

AFP	alphafoetoprotein
ANF	anti-nuclear factor
AR	androgen receptor
ATP	adenosine triphosphate
BSA	bovine serum albumin
CEA	carcinoembryonic antigen
CIEP	counter-immune electrophoresis
CR	complete response
CT	computerised tomography
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
ECG	electrocardiogram
ESR	erythrocyte sedimentation rate
FITC	fluorescein isothiocyanate
FU	fluorouracil
HBcAb	hepatitis B core antibody
HBeAb	hepatitis B e antibody
HBeAg	hepatitis B e antigen
HBsAb	hepatitis B surface antibody
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HLA	human leucocyte antigen
HuIFN- α [Ly]	human lymphoblastoid interferon
IL-2	interleukin-2
IFN	interferon

LAK	lymphokine activated killer
LBBB	left bundle branch block
LV	left ventricular
MEM	modified Eagle's medium
NC	no change
NK	natural killer
PAT	paroxysmal atrial tachycardia
PBC	primary biliary cirrhosis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PD	progressive disease
PEG	polyethylene glycol
PR	partial response
RBBB	right bundle branch block
RIA	radioimmunoassay
RNA	ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
SMA	smooth muscle antibody
SMANCS	styrene maleic acid neocarzinostatin
TAE	trans-catheter arterial embolisation
WCC	white cell count
WHO	World Health Organisation
2,5A	2,5-oligoadenylic acid

INTRODUCTION

Hepatocellular carcinoma (HCC) is the tumour on which the work for this thesis is based. The other less common primary tumours which affect the liver, and metastatic liver tumours, are not discussed.

BACKGROUND

HCC, on a global scale, is one of the commonest malignancies in the world today (Okuda and Beasley, 1982; World Health Organisation Scientific Group, 1983; Cook-Mozaffari and van Rensburg, 1984; Cook, 1985). There is a pronounced geographic variation in the incidence of this tumour, with annual age-adjusted incidence rates ranging from less than four per 100,000 in Northern Europe and North America to greater than 100 per 100,000 in parts of sub-Saharan Africa and the Far East (Waterhouse et al, 1982; Munoz and Linsell, 1982; Cook-Mozaffari and van Rensburg, 1984). In some countries, for example Mozambique and Taiwan, HCC is the most common cause of cancer death, and in China, the most populous country in the world, HCC is the third most frequently observed cancer (Okuda and Beasley, 1982; Munoz and Linsell, 1982). HCC incidence rates increase with age in all parts of the world but the onset of disease tends to be earlier in high incidence areas. In parts of Africa in particular, HCC is a common cause of cancer death in young adults (Munoz and Linsell, 1982).

There are also geographic variations in the clinical presentation and natural history of HCC, though in all regions of the world the prognosis for HCC patients is poor (Okuda et al, 1984). In high incidence areas HCC behaves particularly aggressively and patients

seldom survive more than three months from the time of diagnosis (Kew and Geddes, 1982; Nagasue et al, 1984; Okuda et al, 1984; Falkson et al, 1986).

The treatment of HCC is unsatisfactory. As the tumour most often arises in a cirrhotic liver, and because it is often widespread throughout the liver at the time of diagnosis, surgical resection, the only realistic hope of cure, is usually not possible (Guest and Blumgart, 1987). Currently available cytotoxic agents unfortunately have only limited activity. Most published response rates are less than 20%-30%, and duration of response is usually disappointingly short (Forbes and Williams, 1987). In addition, the toxicity of chemotherapy treatment can be substantial and is capable of diminishing the quality of life of its recipients.

NEW DEVELOPMENTS

In recent years important advances in a variety of the biomedical sciences have occurred, which may in the near future help to dispel some of the gloom usually attached to the subject of HCC.

Epidemiological studies (Szmuness, 1978; Beasley et al, 1981; Beasley, 1982) and the application of the new techniques of molecular biology (Bréchot et al, 1980; Shafritz and Kew, 1981; Bréchot et al, 1982a) have shown a strong association between hepatitis B virus (HBV) infection and HCC development. Indeed, the World Health Organisation (World Health Organisation Scientific Group, 1983) estimate that 80% of all HCCs world-wide may be attributable to HBV infection, and it is this association which predominantly accounts for the marked

geographic variation in HCC incidence previously discussed. Recent reviews have detailed the possible molecular mechanisms whereby HBV infection may ultimately lead to HCC development (Sherman and Shafritz, 1984; Bassendine, 1987; Rogler et al, 1987). It is however also possible that HBV infection leads to HCC development indirectly via the production of cirrhosis (Zaman et al, 1985; Johnson and Williams, 1987), and it remains to be shown conclusively that the HBV is truly oncogenic. Bassendine (1987) has also outlined the other important aetiological factors in HCC development, and has emphasised the complex way in which all factors, including HBV infection, may interact to result in hepatic carcinogenesis.

It is now possible to protect against HBV infection by immunisation. In areas which are endemic for HBV infection (and hence HCC), most infections are acquired in the perinatal period or in early childhood (Beasley, 1982; World Health Organisation Scientific Group, 1983; Beasley and Hwang, 1984; Hsu et al, 1986). Indeed, in Taiwan and other parts of Asia, 40% of all chronic HBV carriers are thought to become infected in the perinatal period (Beasley, 1982). Programmes of combined passive and active immunisation against the HBV, begun shortly after birth, have been shown to protect against subsequent HBV infection (Beasley et al, 1983; Lo et al, 1985; Stevens et al, 1985; Zanetti et al, 1986). If such programmes were instituted in endemic areas, the incidence of chronic HBV infection in the population would fall, and whatever the mechanisms whereby HBV infection results in HCC development, this should eventually lead to a dramatic reduction in HCC incidence. Such vaccination programmes are currently too expensive for most countries where HBV infection is endemic to adopt, but in the near future chemically synthesised polypeptide vaccines and

vaccines produced by recombinant DNA technology may be widely available, and these should be much cheaper than currently available plasma-derived vaccines (Zuckerman, 1985).

The exciting prospect of reducing HCC incidence rates through mass vaccination of new-born infants against HBV infection is still some decades away and in the meantime new methods are needed both for diagnosing HCC at an early stage when curative surgery may be possible, and for treating patients with inoperable tumours.

With the advent of sensitive radioimmunoassays (RIAs) for the detection of serum alphafoetoprotein (AFP) and the development of real-time ultrasonography, it is now possible to non-invasively screen at-risk populations for HCC development. This subject has recently been critically examined by Okuda (1986). While many important questions on population screening for HCC remain to be answered, it is clear that the combined use of serial serum AFP measurements and repeated real-time hepatic ultrasonography represents a major advance in the management of Asian patients with chronic liver diseases, as it allows the early detection of small asymptomatic HCCs at a stage when surgical resection is frequently still possible (Shinagawa et al, 1984; Liaw et al, 1986). This combined approach appears better than AFP screening alone, as small HCCs often do not produce detectable levels of serum AFP (Shinagawa et al, 1984; Kobayashi et al, 1985; Liaw et al, 1986; Ebara et al, 1986) and so may be missed if AFP alone is used for screening. AFP screeing has been very disappointing in the black African population (Purves, 1976), but the value of combined AFP and ultrasound screening remains to be determined, both in this setting and in low HCC incidence areas such as Europe.

More effective and less toxic therapies are needed for inoperable HCC patients, who, for the foreseeable future, will continue to constitute the majority of the HCC population. Several novel modes of cancer therapy now exist whose role in HCC treatment remains to be determined. The interferons (IFNs) are now being extensively investigated in the treatment of a variety of malignancies (Borden, 1984; Krown, 1986; Golomb, 1986). IFNs possess a variety of potent immunomodulatory and antiproliferative properties (Gresser and Tovey, 1978; Borden and Ball, 1981; Stiehm et al, 1982) which make them of potential great importance as anti-cancer agents. Earlier problems of lack of availability have been overcome, as many IFNs are now produced relatively easily by recombinant DNA technology and are now available for clinical study. The production of anti-tumour monoclonal antibodies probably represents the most exciting new development in Clinical Oncology of the last decade. These agents are highly specific, easily and accurately reproduced in bulk, and may enable the targeting of radio-isotopes, cytotoxic drugs and potent cellular toxins to tumour cells. Monoclonal antibodies are now being extensively investigated as potential immunodiagnostic and immunotherapeutic agents (Sikora et al, 1984; Dillman and Royston, 1984; Baldwin and Byers, 1985; Morgan and Foons, 1986; Baldwin and Byers, 1986). Monoclonal antibodies have now been produced to antigens on HCC cell lines (Carlson et al, 1985; Shouval et al, 1985; Hu et al, 1986; Wiedmann et al, in press) and should soon be available in sufficient quantity for evaluation in clinical trials.

THE THESIS

As HCC is relatively rare in this country, and because there is a pronounced geographic variation in the natural history of this tumour, I have begun the thesis with a detailed analysis of the clinical, prognostic, aetiological and pathological features of British HCC patients. I have then reviewed the generally disappointing current status of treatment for HCC and reported on a phase II clinical trial examining the effects of a new anthracenedione, mitozantrone, in HCC treatment. The observed response rate of 27%, while slightly better than most published results with single agent cytotoxic therapies, was disappointing and clearly indicates the need for new approaches to HCC diagnosis and treatment.

In an animal model of HCC I therefore subsequently set out to explore several potential new methods for therapy and diagnosis. In view of the then recent findings of androgen receptors in HCC tumour specimens (Iqbal et al, 1983), the effects of androgenic ablation on HCC growth were studied, and in the light of the recent developments previously discussed, the role of IFNs and anti-HCC monoclonal antibodies in HCC treatment and diagnosis were examined.

CHAPTER ONE

HEPATOCELLULAR CARCINOMA: CLINICAL, AETIOLOGICAL AND PATHOLOGICAL FEATURES IN BRITISH PATIENTS

ABSTRACT

The clinical, prognostic, aetiological and pathological features of 50 consecutive British patients with hepatocellular carcinoma (HCC) have been examined. Presenting symptoms were often vague, and patients were generally in poor condition, ascites and jaundice often being present at diagnosis. The median duration of survival from diagnosis was only seven weeks. Only initial performance grade and the presence or absence of cirrhosis significantly affected prognosis. Serum alphafoetoprotein was positive by counter-immune electrophoresis in only 40% of patients, but 76% were positive when radioimmunoassay was used. Cirrhosis was present in 39 patients (78%) and was usually cryptogenic (13), hepatitis B virus (HBV) - related (9) or alcoholic (7) in origin. Serum HBsAg was detected in 10 patients (20%), and of the remainder, 21% had serological evidence of past HBV exposure. None of nine serum HBsAg negative tumour specimens had detectable HBV-DNA integration into the tumour cell genome. Liver cell dysplasia was noted in 53% of patients with liver biopsies predating the diagnosis of HCC, and in 79% of biopsies taken at the time of HCC diagnosis. The commonest histological pattern was trabecular (70%). Other forms were rare; the fibrolamellar pattern was only seen in two patients.

In British patients, HCC is usually diagnosed late and is usually associated with established cirrhosis. It is rapidly fatal, particularly in cirrhotic patients and in those with poor initial performance score. In 40% it is associated with serological evidence of past or present HBV infection. The relationship between liver cell dysplasia and the development of HCC merits further study.

INTRODUCTION

As previously mentioned, HCC is an uncommon cancer in Northern Europe and North America but is frequently seen in sub-Saharan Africa and the Far East. The clinical features in high incidence areas are well described (Lai et al, 1981; Kew and Geddes, 1982; The Liver Cancer Study Group of Japan, 1984). HCC, as it is seen in the United Kingdom however, often seems to be diagnosed late, and most patients die within a few weeks of diagnosis. A variety of pertinent features in 50 consecutive British patients with histologically proven HCC have therefore been analysed in order to determine whether some common presenting clinical features may suggest HCC development, and whether some simple clinical and laboratory parameters may be of prognostic value.

Possible aetiological factors have also been examined. Using serological radioimmunoassays and radiolabelled DNA probes to detect viral DNA in tumour tissue, the relationship between chronic HBV infection and HCC has been particularly studied. Finally, hepatic histology has been reviewed.

PATIENTS AND METHODS

All patients were seen at the Royal Free Hospital, London, between January 1978 and December 1984. Forty-one patients (82%) were born in Britain and the remaining nine patients (18%), were immigrants (one from Africa, two from Asia, two from the Far East and three from the West Indies). Information was available on age, sex, presenting symptoms, performance status (WHO, 1979), physical signs, presenting

haematological and biochemical parameters, the results of hepatic imaging techniques, duration of survival,and the cause of death.

Serum was analysed for alphafoetoprotein (AFP) by radioimmunoassay (RIA) (Hoechst Laboratories, UK) and counter-immune electrophoresis (CIEP). Carcinoembryonic antigen (CEA) concentration was measured by RIA (Abbot Laboratories, UK). Sera were tested for HBsAg, HBsAb and HBcAb using commercially available RIAs (all Abbot Laboratories, UK). HBsAg positive sera were analysed for HBeAg and HBeAb (Abbot) and also for serum HBV-DNA, using a modification (Karayiannis et al, 1985) of the dot hybridisation technique (Weller et al, 1982). In 10 patients tumour tissue was available for analysis of HBV-DNA sequences by southern blotting and hybridisation to a (^{32}P) HBV-DNA probe (Monjardino et al, 1982; Fowler et al, 1984).

Cirrhosis was diagnosed by histological examination of non-tumour tissue, and the aetiology defined on clinical, serological and histological grounds (see below). Liver cell dysplasia (Anthony et al, 1973) was sought in all biopsy specimens, including specimens pre-dating the development of HCC. The histological and cytological variants of HCC were characterised (Anthony, 1987).

Statistical analyses were performed using the Chi-squared test with Yates correction and the log-rank test as appropriate. Survival curves were produced by the Kaplan-Meier method.

RESULTS

There were 37 men and 13 women (male:female, 2.8:1). The median age was 58 years (range 16-77 years); 12 patients (23%) were less than 40 years old.

Symptoms and physical signs at presentation

In four patients the diagnosis of HCC was not suspected antemortem but was made at autopsy. In two patients the diagnosis was made while the patient was asymptomatic; one at cholecystectomy, and the other while undergoing routine liver biopsy for assessment of primary biliary cirrhosis (PBC). The major presenting symptoms in the remaining 46 patients were malaise (70%), anorexia (60%) and abdominal pain (55%) (Table 1 (i)). Pain was usually in the right hypochondrium or epigastrium, and of mild to moderate severity. It was chronic and no patient presented as an acute emergency due to tumour rupture. Abdominal swelling, either due to ascites or to a palpable tumour mass, was noted by 37% of patients, and 18% presented either with jaundice or gastrointestinal bleeding, the latter always proven by endoscopy to be due to variceal rupture.

The presenting performance status of the 46 patients diagnosed in life was graded using World Health Organisation (WHO) criteria (WHO, 1979). Only five (11%) patients were unrestricted in normal activities and therefore in the grade 0 category. Most patients fell into the grade 1 category (33%) while 22% and 26% respectively fell into grades 2 and 3. Four patients (8%) were completely disabled, bed-bound and unable to perform any self-care, and therefore of grade 4 status.

Table 1 (i)

PRESENTING SYMPTOMS OF HEPATOCELLULAR CARCINOMA IN BRITISH PATIENTS.

Symptom	% with symptom
Malaise	70
Anorexia	60
Abdominal pain	55
Weight loss	47
Diffuse abdominal swelling	27
Jaundice	18
Gastrointestinal bleeding	18
Pruritis	12
Palpable mass	10
Ankle oedema	10
Cough or dyspnoea	6
Deep vein thrombosis	4
No symptoms	4
Fever	2

The physical signs most commonly seen at presentation are listed in Table 1 (ii). In three of the 46 patients tense ascites rendered abdominal palpation impossible. Hepatomegaly was noted in 40 (93%) of the remaining 43 patients. The liver was invariably hard and in 20% was tender. An arterial bruit was heard over the enlarged liver in three patients (7.5%). Ascites was detectable in 43%. It was usually of mild or moderate severity but in five patients (11%) demanded immediate paracentesis. Splenomegaly was noted in 25%, all cases being cirrhotic. Spider naevi were seen in 35% of patients and jaundice noted in a similar proportion. Hepatic encephalopathy was noted in seven patients (15%), being of grade I severity in six and grade II in the other. In two patients the encephalopathy was ascribed to gastrointestinal bleeding and in five was thought to represent hepatocellular failure.

Standard haematological and biochemical tests (Tables 1 (iii) & 1 (iv))

The erythrocyte sedimentation rate (ESR) was abnormal in 27 of the 32 patients tested, five patients (16%) having values greater than 100 mm in the first hour. The mean haemoglobin concentration was 12.7 g/dl. Three of the 46 patients (6.5%) had haemoglobin values above the upper limit of normal in the absence of transfusion, but none had a raised haematocrit. Five patients (11%) had a haemoglobin concentration less than 10 g/dl at presentation, three of whom had recent gastrointestinal bleeding. Prothrombin time was abnormal in 38 of 44 patients, the mean prolongation being almost four seconds. One patient, who had no initial clotting studies, developed small intestinal venous thrombosis, requiring surgery and subsequent anticoagulant therapy.

Table 1 (ii)

MOST COMMON PRESENTING PHYSICAL SIGNS IN BRITISH PATIENTS WITH HEPATOCELLULAR CARCINOMA.

Sign	% with sign
Hepatomegaly	93
- % with tenderness	20
- % with bruit	7.5
Ascites	43
Splenomegaly	25
Jaundice	35
Spider naevi	35
Encephalopathy	15

A single biochemical abnormality in liver function tests was seen in four patients (9%); three showed a raised serum alkaline phosphatase concentration, and one an increase in serum aspartate transaminase. Among the remaining patients, no particular pattern (i.e. 'hepatitic' or 'cholestatic') predominated. Serum aspartate transaminase and alkaline phosphatase were each elevated in 90% of patients. At presentation 76% of patients showed a raised serum bilirubin concentration, the level in 17 (37%) exceeding 50 μmol/l. The mean serum albumin concentration was 35 g/l, with 24% of patients having levels less than 30 g/l. Six of 45 patients (13%) had raised corrected serum calcium levels. No patient had obvious bone metastases and none had symptomatic hypercalcaemia. The serum phosphate concentration was reduced in only one of these patients, suggesting possible hyperparathyroidism. Random plasma glucose concentrations were measured at presentation in 35 subjects, and none had evidence of hypoglycaemia. Clinical hypoglycaemia was not a problem subsequently.

Presenting serum AFP and CEA concentrations

AFP was measured in the serum by CIEP (lower limit of sensitivity around 500 IU/ml) (Eleftheriou et al, 1977) in all patients, and by RIA (lower limit of sensitivity <10 IU/ml) in 42 patients. Serum AFP was positive by CIEP in 20 patients (40%) and negative in 30 (60%). A serum AFP > 10 IU/ml was detected by RIA in 32 of the 42 patients tested (76%). The presence or absence of either cirrhosis or serum HBsAg had no effect on AFP positivity rates when measured by CIEP (Yates corrected Chi-squared test; $p>0.05$).

Table 1 (iii)

INITIAL HAEMATOLOGICAL VALUES IN BRITISH HCC PATIENTS DIAGNOSED ANTE-MORTEM.

TEST (units)	NO. TESTED	% ABNORMAL	MEAN	RANGE	NORMAL RANGE
ESR (mm in 1st hour)	32	84	56	2-122	0-15
Haemoglobin (g/dl)	46	71	12.7	7.3-17.7	14.0-17.0 (Male) 12.0-15.5 (Female)
Haematocrit (1/1)	38	50	0.39	0.25-0.53	0.4-0.54 (Male) 0.37-0.47 (Female)
White cell count ($\times 10^9$/1)	46	24	7.5	2.5-28.0	4-11
Platelet count ($\times 10^9$/1)	44	43	197	57-460	150-350
Prothrombin time (seconds different from control)	44	90	+3.6	(-1)-(+27)	-

Table 1 (iv)

PRESENTING BIOCHEMICAL DATA IN BRITISH HCC PATIENTS DIAGNOSED ANTE-MORTEM (n = 46).

TEST (units)	No. TESTED	% ABNORMAL	MEAN	RANGE	NORMAL RANGE
Bilirubin (μmol/l)	46	76	53	6-442	5-17
Alkaline phosphatase (IU/l)	46	90	281	90-1083	30-130
Albumin (g/l)	46	26	35	20-52	30-50
Aspartate transaminase (IU/l)	46	90	189	21-2100	5-40
Calcium (corrected) (mmol/l)	45	13	2.45	2.17-2.88	2.12-2.62
Glucose (random) (mmol/l)	35	77	6.9	4.2-14.0	2.9-8.0

Serum CEA concentrations were measured in 21 HCC patients at diagnosis. Only three (14%) had raised levels, and in only one patient was CEA concentration in the 'tumour range' of greater than 20 μg/l.

Hepatic imaging techniques

An isotope liver scan (using technetium sulphur colloid) was performed in 27 patients. Generalised patchy and reduced uptake of radiocolloid was reported in 12 patients (44%). Definite filling defects, indicating space occupying lesions, were seen in the livers of 20 of the 27 patients (74%). In six patients (22%) patchy uptake of radiocolloid precluded a definite diagnosis of hepatic filling defects and in the remaining patient (4%) a normal scan was reported. Coeliac or selective hepatic angiography was performed in 30 patients. Tumour circulation was noted in 29 patients (97%) and in only one patient was the hepatic vasculature reported as normal. Abdominal CT scan, with and without intravenous contrast enhancement, showed hepatic filling defects in 23 of the 24 (96%) patients in whom it was performed, and was frequently used in order to selectively target biopsy needles into the filling defects.

Hepatocellular carcinoma: aetiology and relationship to cirrhosis (Table 1(v))

In 39 patients HCC was associated with underlying cirrhosis (78%). In six patients (12%) the liver was non-cirrhotic and in five patients (10%) the state of the underlying non-malignant liver was not known.

The aetiology of the underlying cirrhosis was classified as follows:- Alcoholic cirrhosis, defined as the presence of cirrhosis in a subject

with a regular alcohol consumption of >80g per day and/or hepatic histological features compatible with alcohol abuse (Scheuer, 1980), was present in 18%. Cryptogenic cirrhosis, diagnosed in serum HBsAg negative subjects with no history of alcohol abuse, and who had no autoantibodies (anti-nuclear factor (ANF) < 1:40 and smooth muscle antibody (SMA) < 1:40) or histopathological features characteristic of other liver diseases, was observed in 33%. Cirrhosis associated with auto-immune chronic active hepatitis, diagnosed in the presence of an ANF or SMA $\geqslant$ 1:40 was present in 8%, and cirrhosis associated with HBV, diagnosed if serum HBsAg was detected by RIA, was seen in 23%. Other types of cirrhosis were diagnosed on histological features (Scheuer, 1980). In the group of non-cirrhotic patients, and in those where the state of the underlying non-malignant liver was not known, usually no recognised aetiological factor could be detected. Two tumours however arose in young women in relationship to pregnancy, one becoming apparent in the third trimester of pregnancy and the other six months after a normal full-term pregnancy.

HCC: relationship to HBV infection (Table 1 (vi))

Ten of 50 patients were positive for serum HBsAg. Of these patients nine were tested for HBeAg and HBeAb, and seven for serum HBV-DNA sequences. Seven patients were positive for HBeAb and negative for HBeAg and two were positive for HBeAg and negative for HBeAb. Serum HBV-DNA was detectable in three of the seven patients examined, two being HBeAb positive. Thirty-three of the 40 patients negative for serum HBsAg were tested for both HBsAb and HBcAb. Twenty-six patients (79%) had no serological evidence of past infection with HBV, six (18%) had both antibodies, and one patient (3%) had HBcAb alone. The distribution of HBV antibodies between the various types of liver

Table 1 (v)

AETIOLOGY OF UNDERLYING LIVER DISEASE IN THE 39 HCC PATIENTS WITH CIRRHOSIS.

Aetiology of cirrhosis	N	%
Cryptogenic	13	33
HBsAg +ve	9	23
Alcoholic	7	18
Primary Biliary Cirrhosis	4	10
Auto-immune	3	8
Haemochromatosis	2	5
α1-antitrypsin deficiency	1	3

Table 1 (vi)

HBeAg, ANTI-HBe AND SERUM HBV-DNA STATUS OF NINE SERUM HBsAg POSITIVE HCC PATIENTS.

HBeAg	anti-HBe	serum HBV-DNA
-	+	-
-	+	-
-	+	-
-	+	+
-	+	+
-	+	NT
-	+	NT
+	-	-
+	-	+

NT = not tested

disease encountered in the study population is shown in Table 1 (vii). Amongst those patients with serum HBsAg negative cirrhosis, serological evidence of past HBV infection was only seen in those with cryptogenic (four of 11 tested) or alcoholic cirrhosis (two of five tested).

Nine patients, all serum HBsAg negative but two with serological evidence of past HBV infection, had tumour specimens available for HBV-DNA analysis. No patient had evidence of episomal or integrated HBV-DNA sequences.

Cause of Death, duration of survival, and prognostic factors in HCC

Thirty-three of the 46 patients diagnosed in life received chemotherapy, usually with either doxorubicin or mitozantrone. Nine patients (20%) were considered too ill for any form of treatment, and only four patients (9%) were considered suitable for tumour resection. By the end of February 1985, 37 of the 46 patients had died, six were still alive (median duration of survival to date 60 weeks from diagnosis, range 12-120 weeks) and three had been lost to follow-up. The causes of death in HCC patients are listed in Table 1 (viii). The majority of patients (20/37, 54%) died of disease progression, shown by increasing hepatocellular failure and generalised wasting. Thirteen patients (35%) died from variceal haemorrhage. Other causes of death were unusual, and no patient died of tumour rupture.

Table 1 (vii)

DISTRIBUTION OF HBV ANTIBODIES AMONGST SERUM HBsAg NEGATIVE HCC PATIENTS.

State of non-malignant liver	Total No in study	No tested	HBV antibodies	
			Present*	Absent
Cryptogenic cirrhosis	13	11	4	7
Alcoholic cirrhosis	7	5	2	3
Primary biliary cirrhosis	4	4	0	4
Auto-immune type cirrhosis	3	3	0	3
Haemochromatosis	2	2	0	2
α 1-antitrypsin deficiency	1	1	0	1
Normal	6	5	1	4
Not known	4	2	0	2

*anti-HBc alone or anti-HBc and anti-HBs

Table 1 (viii)

CAUSES OF DEATH IN HEPATOCELLULAR CARCINOMA.

Cause	N	%
Disease progression	20	54
Variceal haemorrhage	13	35
Treatment related	3	8
Unrelated to HCC	1	3

The median duration of survival of all patients diagnosed in life was seven weeks from diagnosis (Figure 1 (i)). By using the log-rank test to compare survival curves, the effects on survival duration of age (greater or less than 50 years), sex, serum HBsAg, serum AFP (positive or negative by CIEP), initial performance status (WHO grades 0 and 1 v WHO grades 2-4) and cirrhosis were studied. Only the presence or absence of cirrhosis ($p = 0.002$, Figure 1 (ii)) and initial performance status ($p = 0.0001$, Figure 1 (iii)) had prognostic value.

Histopathological features

Nineteen of the 50 patients had had liver biopsies (in 13 single and in six multiple) pre-dating the histological diagnosis of HCC. Liver cell dysplasia was noted in the biopsies of 10 of these 19 patients (53%) (Figure 1 (iv)). The median time interval between the observation of liver cell dysplasia and the diagnosis of HCC was 37.5 months (range one day - 15.5 years). In the 50 liver specimens diagnostic of HCC, non-malignant liver was also seen in 38 cases, and of these, liver cell dysplasia was present in non-tumorous areas in 30 patients (79%). No significant differences (Yates corrected Chi-squared test) could be found in the overall observed frequency of liver cell dysplasia between HBsAg positive and negative patients (8/9 (89%) and 28/36 (78%) respectively) and between cirrhotic and non-cirrhotic patients (30/39 (77%) and 4/6 (66%) respectively).

The histological patterns and cytological variants of HCC observed are listed in Table 1 (ix). The trabecular histological form occurred in 35 of 50 patients (70%). Tumours with this pattern were usually moderately or well differentiated (Edmondson, 1958) and of the

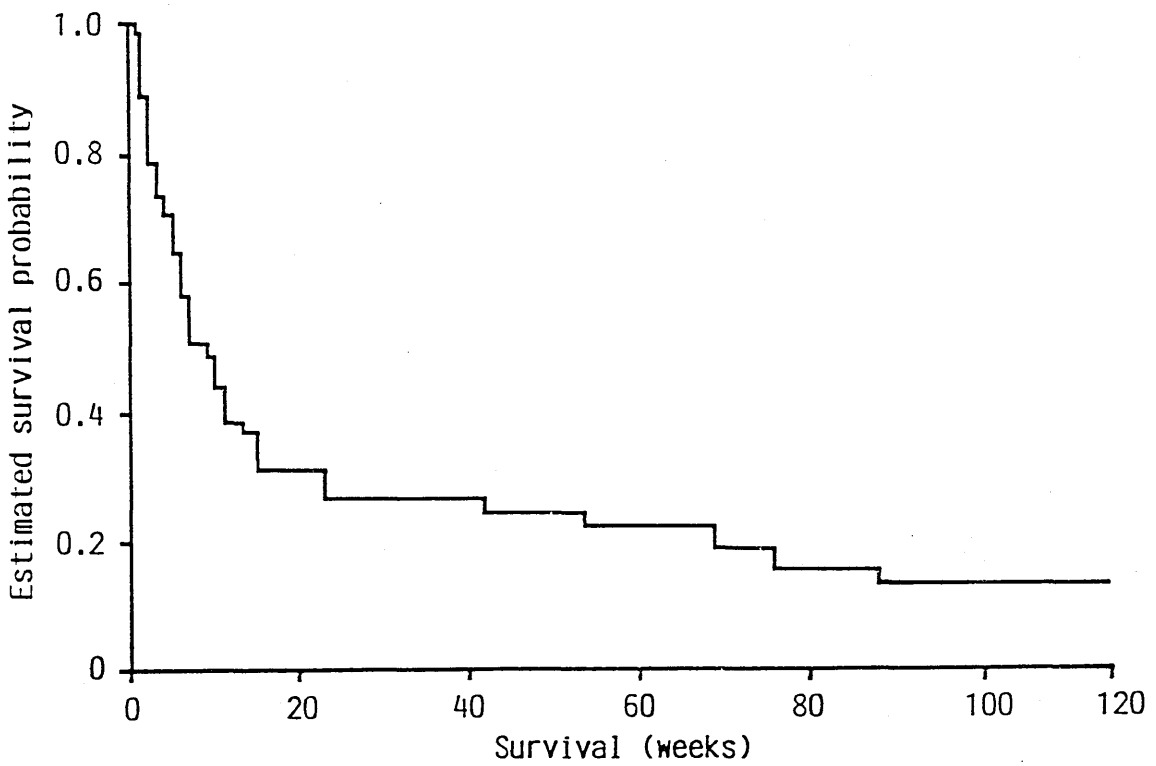

Figure 1 (i)

KAPLAN-MEIER SURVIVAL CURVE OF THE 46 HCC PATIENTS DIAGNOSED ANTE-MORTEM. Duration of survival was calculated from the time of HCC diagnosis.

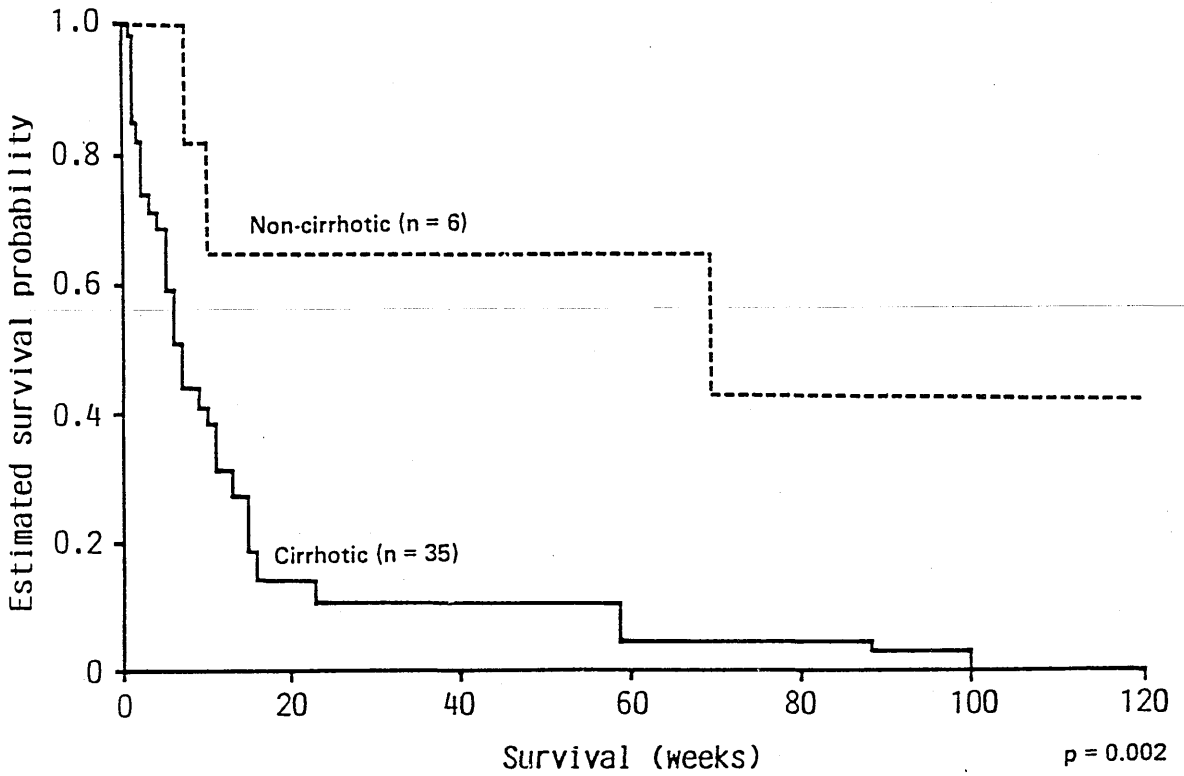

Figure 1 (ii)

KAPLAN-MEIER SURVIVAL CURVES OF CIRRHOTIC AND NON-CIRRHOTIC PATIENTS WITH HCC. Survival was significantly longer in the non-cirrhotic group ($p = 0.002$, log-rank test).

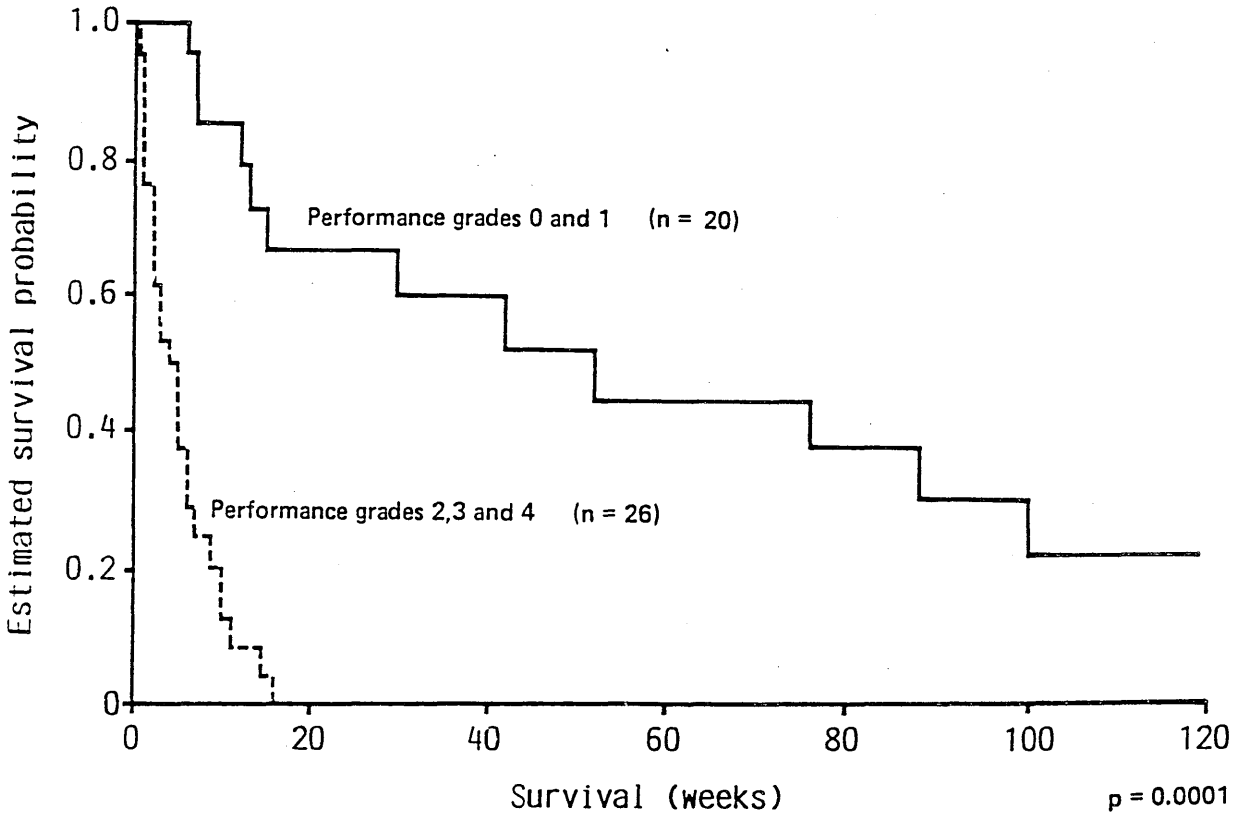

Figure 1 (iii)

KAPLAN-MEIR SURVIVAL CURVES OF HCC PATIENTS OF VARYING INITIAL WHO PERFORMANCE STATUS. Patients of WHO grades 0 and 1 survived significantly longer than patients of WHO grades 2 - 4 ($p = 0.0001$, log-rank test).

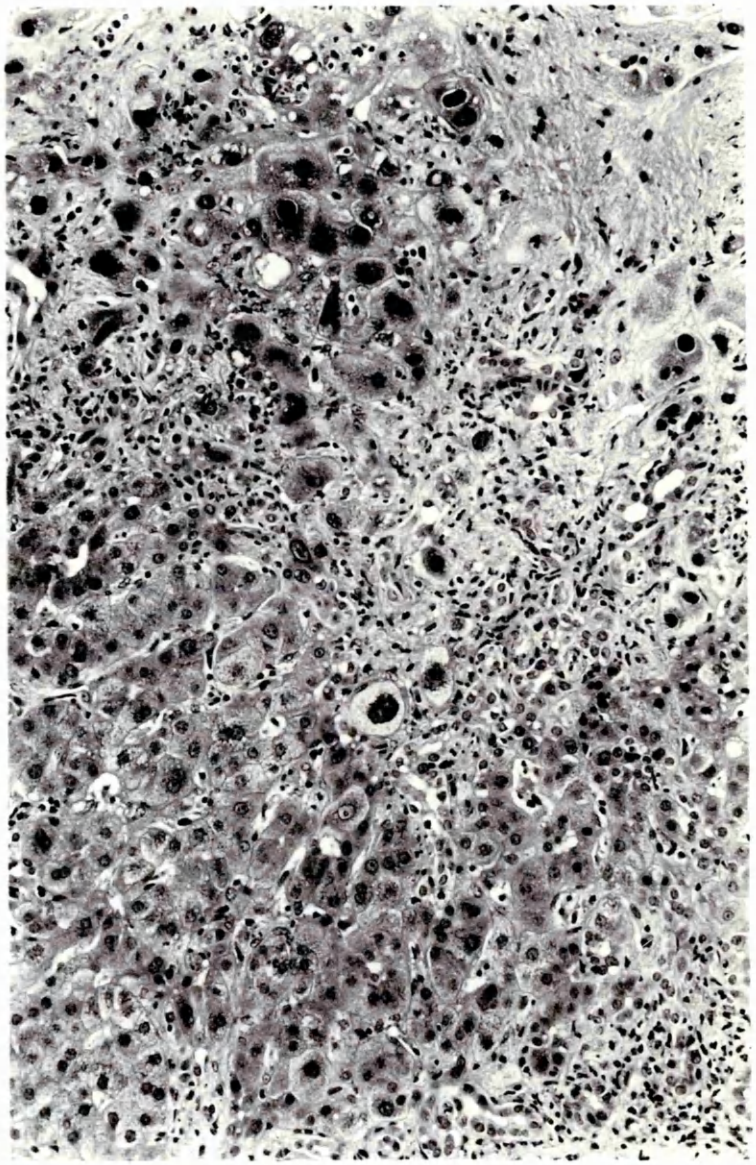

Figure 1 (iv)

LIVER CELL DYSPLASIA. Hepatocytes are of variable size and their nuclei are enlarged, irregular and hyperchromatic (haematoxylin and eosin) x 40.

Table 1 (ix)

HISTOPATHOLOGICAL ANALYSIS OF 50 HCCs ARISING IN BRITISH PATIENTS.

HISTOLOGICAL PATTERN	N	CYTOLOGICAL VARIANT				
		HEPATIC	CLEAR CELL	PLEOMORPHIC	MIXED	UNCLASSIFIABLE
Trabecular	35	26	3	2	3	1
Pseudoglandular (Adenoid) (Acinar)	2	2	0	0	0	0
Scirrhous	3	2	0	1	0	0
Mixed	4	3	1	0	0	0
Fibrolamellar	2	2	0	0	0	0
Unclassifiable	4	1	0	0	0	3

hepatocytic cytological variant. Fibrolamellar carcinoma, which has been associated with a good prognosis (Berman et al, 1980), was seen in only two patients, both young adults (ages 16 and 26) with non-cirrhotic livers. Four carcinomas could not be classified. In two the tumour specimen was either too small or too necrotic, and in the other two patients the histological pattern observed could not be matched with any of the categories described by Anthony (1987). One of these tumours consisted of a mixture of hepatocellular and duct-like elements in which the former displayed trabecular, acinar and scirrhous areas, and in the other tumour, trabecular, acinar and scirrhous areas were once again noted, in a tumour specimen which was mostly necrotic. Bile secretion, which is considered pathognomonic of HCC (Anthony, 1987), was observed in 21 tumours (44%), the majority (16/21) being of trabecular pattern.

DISCUSSION

This series provides the most detailed aetiological, clinicopathological and prognostic analysis so far undertaken on purely British HCC patients.

The male predominance and the age distribution of HCC patients is similar to that reported from other low incidence areas (Ihde et al, 1974; MacSween, 1974; Johnson et al, 1978a; Chlebowski et al, 1984a) and from recent series from the Far East (The Liver Cancer Study Group of Japan, 1984; Lin et al, 1984), though the male:female ratio of 2.8:1 is lower than that previously reported for the U.K. (MacSween, 1974; Johnson et al, 1978a). The cause of this difference is unclear

and may simply reflect differing referral patterns between the centres. It appears to be unrelated to oral contraceptive usage as only four of the women were in the reproductive age group, and only two had used oral contraceptives. Though HCC in low incidence areas is often considered a tumour of older people, approximately a quarter of British patients were under the age of 40 years at diagnosis.

The presenting symptoms of British HCC patients were similar to those previously reported from the United Kingdom (MacSween, 1974), North America (Chlebowski et al, 1984a), and the Far East (The Liver Cancer Study Group of Japan, 1984; Nagasue et al, 1984). Symptoms were generally non-specific and of little diagnostic value. However, upper abdominal pain, particularly in the right hypochondrium, was a presenting feature in 55% of patients, and this symptom, in an at-risk patient, should arouse the suspicion of HCC development. Presentation as an acute abdominal catastrophe due to tumour rupture, although found in 10% of the South African black HCC population (Kew and Geddes, 1982), was not observed. Hepatomegaly, ascites and jaundice were the most common physical signs. The liver was usually very firm and often tender, but a hepatic bruit, advocated as a common physical sign in HCC, even in Britain (Kew et al, 1971), occurred in only 7.5% of patients.

Clinical condition at diagnosis was often poor, and 34% of patients were of WHO performance grade 3 or worse on presentation. Thus British HCC patients are often diagnosed late. To prevent this, the screening of at-risk populations for the early development of HCC is often advocated, whether this be by serial serum AFP measurements (for review see Warnes and Smith, 1987) or ultrasonic liver scanning

(Shinagawa et al, 1984; Liaw et al, 1986). However, our data suggest that screening programmes would have a limited impact on the course of the disease in this country, as 46% of patients presented de novo with established HCC and without previously diagnosed chronic liver disease, and so would not have been included in any screening programme.

Standard haematological and biochemical tests were often abnormal but showed no specific diagnostic pattern. Para-neoplastic phenomena of HCC (Margolis and Homcy, 1972; Kew and Dusheiko, 1981) were uncommon. At diagnosis, serum AFP concentration was elevated in 76% and 40% of patients respectively when measured by RIA and by the less sensitive CIEP technique. These results are similar to those of other series from low incidence areas (Johnson et al, 1978b; Kew and Newberne, 1982). An AFP concentration of greater than 1000 IU/ml, often considered diagnostic of HCC (Johnson et al, 1978b), was found in 38%. As noted previously (Melia et al, 1981a), the measurement of serum CEA was of no diagnostic help.

Hepatic angiography and CT scanning proved to be highly sensitive and equally effective diagnostic techniques. In addition hepatic angiography provided useful information in determining tumour resectability and portal vein invasion by tumour, and allowed arterial embolisation of tumour masses and hepatic arterial chemotherapy if desired. Isotope liver scanning, as previously noted (Kew et al, 1971; Bryan et al, 1977), was less sensitive, demonstrating hepatic filling defects in approximately 75% of patients.

The majority of HCCs arose in a cirrhotic liver. This finding agrees with results from other geographic regions (Kew and Popper, 1984). The percentage observed (78% of the total series and 87% of the series in whom the state of the underlying non-malignant liver could be fully assessed) is similar to that previously reported for largely British subjects (Sharpstone et al, 1972; MacSween, 1974; Johnson et al, 1978a). The most commonly observed types of cirrhosis were cryptogenic, HBsAg positive and alcoholic. In each type there was a marked male predominance (cryptogenic cirrhosis: 11 of 13; HBsAg positive cirrhosis: nine of nine; alcoholic cirrhosis: six of seven), and of the serum HBsAg negative types of cirrhosis, only those of cryptogenic or alcoholic types showed serological evidence of past HBV infection. The propensity of cryptogenic, alcoholic and serum HBsAg positive cirrhotic patients to develop HCC is well described (MacSween and Scott, 1973; Kew and Popper, 1984). The small number of patients with haemochromatosis and HCC in the current study probably reflects the relative rarity of this condition in the Royal Free Hospital patient population.

Approximately 20% of British HCC patients were found to have a positive serum HBsAg, and in the remainder positive tests for HBsAb and/or HBcAb were found in 21%. These findings are in broad agreement with those of Bassendine et al (1979). However, in contrast to the findings of Bréchot et al (1982a, 1983b), detectable HBV-DNA integration into the tumour cell genome of serum HBsAg negative patients could not be found, and it is therefore difficult to implicate the HBV in the development of tumours in this group, which comprise the majority of British patients. As has been previously

suggested (Fowler et al, 1986), it is possible that in these patients HBV-DNA integration may have occurred at the onset of malignant transformation and that subsequently integrated sequences have become undetectable, or it may be that other factors, such as the presence of cirrhosis per se (Kew and Popper, 1984; Zaman et al, 1985; Johnson and Williams, 1987) are linked to HCC development in these patients. Unfortunately no tumour tissue from serum HBsAg positive cases was available for HBV-DNA probing.

The diversity of patients with respect to age, gender, presence or absence and type of cirrhosis, alcohol consumption, and evidence of past or present HBV infection, emphasises the complex, multifactorial, and probably interactive relationships involved in the aetiology and pathogenesis of HCC, particularly in low incidence areas (Cook-Mozaffari and van Rensburg, 1984; Kew and Popper, 1984; Zaman et al, 1985; Bassendine, 1987).

The median duration of survival from the time of diagnosis of HCC was only seven weeks, emphasising the poor prognosis of this condition. For many reasons, including differing patient characteristics and uncertainty over whether survival times are taken from the onset of symptoms or the time of diagnosis, an accurate comparison with other series is difficult. However, this survival time is considerably worse than that seen in previous series of predominantly European (Davidson et al, 1974; Melia et al, 1984) and American (Chlebowski et al, 1984a) patients and approaches that seen in the rural South African black population (Kew and Geddes, 1982) and in a recent large series of untreated patients from the Far East (Nagasue et al, 1984). The initial poor performance status of the majority of British

patients would seem the most likely reason for the poor survival figures observed, as presenting performance status proved to be a very powerful prognostic indicator of survival in this population. The presence or absence of cirrhosis also proved to be of prognostic importance. This confirms results from predominantly low incidence areas (Johnson et al, 1981; Melia et al, 1984) and is in contrast with the findings of series from high incidence areas, where cirrhosis has little or no effect on the course of the disease (Primak et al, 1975; Kew and Geddes, 1982; Nagasue et al, 1984). Age at diagnosis had no effect on survival in our HCC population though it did have in other series from similar areas (Johnson et al, 1981; Chlebowski et al, 1984a). This difference probably arises because of the larger percentage of cirrhotic patients in the current series compared to the others, as it seems likely that the observed improved survival in younger patients is explained by the fact that tumours in this age group are more likely to arise in a non-cirrhotic liver (Chlebowski et al, 1984a). The presence or absence of serum HBsAg did not significantly affect the prognosis of British patients, and this is in agreement with studies from other regions (Chlebowski et al, 1984a; Falkson et al, 1986).

The microscopic features of HCC were in general as described by Anthony (1987), the majority being of the trabecular pattern. The importance of liver cell dysplasia in the non-malignant liver remains controversial. Though a potential pre-malignant lesion (Anthony et al, 1973; Ho et al, 1981; Roncalli et al, 1986), its role in this regard has been disputed (Cohen et al, 1979; Nakashina et al, 1983; Cohen and Berson, 1986) but not resolved. The incidence of liver cell dysplasia in the current series was surprisingly higher than that

reported from other areas (Anthony et al, 1973; Peters, 1976; Cohen et al, 1979; Ho et al, 1981; Nakashina et al, 1983), being 53% in those patients with biopsies predating the diagnosis of HCC, and rising to 79% in biopsies taken at the time of HCC diagnosis. No relationship between serum HBsAg and liver cell dysplasia could be demonstrated in British HCC patients. Prospective controlled studies of the fate of patients with liver cell dysplasia and no evidence of HCC development are required.

CONCLUSIONS

HCC as seen in the United Kingdom is a disease of male predominance with a peak incidence in the sixth decade. It usually arises in a cirrhotic liver; approximately 20% of all cases are associated with a positive serum HBsAg.

HCC presents late and patients are generally in poor clinical condition when diagnosed. The median survival time from diagnosis of HCC was only seven weeks. Though effective screening systems to detect HCC at an early stage may result in more effective treatment for some patients, our results show that almost one half of British HCC patients present *de novo* with HCC and would have escaped screening.

The role of liver cell dysplasia in hepatocarcinogenesis merits detailed study.

CHAPTER TWO

THE TREATMENT OF HEPATOCELLULAR CARCINOMA

This review deals only with the specific treatment of HCC. The relief of the symptoms of HCC is not discussed, though this is clearly an area of great importance.

PROBLEMS IN ASSESSING HCC TREATMENTS

The accurate evaluation of clinical trials of HCC treatments is hindered by many problems. The major drawbacks to individual studies, particularly those from areas of low HCC incidence, are usually the small size of the study population and the lack of untreated or other relevant control groups. The absence of a universally agreed and applied definition of what constitutes "response to treatment" and the pronounced geographic variation in the natural history of HCC both combine, along with variable patient selection criteria, to render between-trial comparisons of specific HCC therapies difficult and unwise.

TREATMENT CHOICES IN HCC

Treatment choices may be broadly categorised as shown in Table 2 (i). Surgical resection offers the only realistic hope of cure, other therapies being palliative. As resection rates are generally low, medical (cytotoxic) therapy is the backbone of treatment. Many of the treatments listed are experimental and require much further evaluation.

Table 2 (i)

TREATMENT CHOICES IN HCC.

SURGICAL THERAPY

- hepatic resection
- hepatic transplantation
- ligation of the hepatic artery or portal vein

RADIATION THERAPY

- external irradiation
- "internal" irradiation

CYTOTOXIC CHEMOTHERAPY

- single agent
- multiple agent
- (intravenous or intra-arterial routes of administration?)

IMMUNOTHERAPY

- levamisole
- interferons
- anti-HCC antibodies
- lymphokine-activated killer cells

HORMONAL THERAPY

- tamoxifen
- cyproterone
- progestins

COMBINATION THERAPIES

THE SURGICAL TREATMENT OF HCC

Surgical resection in HCC is traditionally felt possible only when the tumour is confined to one liver lobe, does not involve major vascular structures, and arises in a non-cirrhotic liver. Some surgeons nowadays will however attempt curative resection in cirrhotic patients if liver function is good (Lee et al, 1982; Li et al, 1985; Bismuth et al, 1986; Kinami et al, 1986), and in highly selected non-cirrhotic patients, specialist hepato-biliary surgeons may achieve apparently curative resections even in those with extensive tumours invading large blood vessels (Søreide et al, 1985).

Resectability rates vary from one geographic region to another. Resectable tumours are found in less than 5% of black Africans with HCC (Harrison et al, 1973; Kew, 1982), whereas in Japanese patients, resection rates are substantially higher and appear to be rising, probably mainly because HCC is being diagnosed earlier in its natural history by screening tests. Between 1968 and 1977 the Japanese Liver Cancer Study Group reported a resection rate of 11.9% in 2411 patients (Okuda et al, 1980), and for the period 1978 to 1979 this figure had risen to 26.6% (Tobe et al, 1982). In these studies patients with resectable tumours had one and five year survival rates of 33% and 12% respectively, and Okuda et al (1984) have recently reported a one year survival rate of 62% for resectable patients, this figure being significantly better than that achieved by comparable medically treated patients. In China, similar one year survival rates are being reported (Li et al, 1985), and resection rates of 60% have been reported in patients with sub-clinical HCC detected by screening, with five year survival rates of over 70% in resected patients with sub-clinical disease (Tang, 1985). In the West, resection rates are

variably reported. In the older literature, resection rates of less than 5% are generally reported (Longmire et al, 1966; Bengmark et al, 1971; Linder et al, 1974), though in highly selected groups resection rates of 23% have been reported (Mabogunje et al, 1975). More recently Lim and Bongard (1984) have reported a 25% resection rate in 86 patients, predominantly Chinese Americans, though in a series of 46 British patients seen at the Royal Free Hospital, London between 1978 and 1984, the resection rate was only 9% (see Chapter 1). This disappointing figure may reflect a referral bias in the population studied. Table 2(ii) summarises the results of several recent series dealing with hepatic resection in HCC.

In several centres orthotopic liver transplantation has been performed on patients considered unsuitable for hepatic resection, either because of poor liver function or because of widespread involvement of the liver by tumour. Such patients must satisfy the usual criteria of suitability for hepatic transplantation (van Thiel et al, 1984) as well as have no tumour invasion of major blood vessels and no extrahepatic spread of tumour. In those centres where transplantation is available, the few results to date suggest that it may have a palliative role in HCC treatment (Rolles et al, 1984; Pichlmayer et al, 1984). In Rolles' series (1984), 32 of 40 patients with primary hepatic tumours survived for greater than 12 months post transplantation. Long-term survivors and apparent cures have been seen at other centres (Pichlmayer et al, 1984; Krom et al, 1984), and Scharschmidt (1984), reporting the combined experience of four centres, found a 26% one year survival rate in a group of 139 patients transplanted for hepatic malignancy, 62% of whom had HCC. Tumour recurrence is a major problem in long-term survivors and in the

Table 2 (ii)

HEPATIC RESECTION FOR HCC: RESECTION RATES AND RESULTS.

Author	Country	Total No. Cases Seen	No. of Resections (% Total Cases)	No. of Cirrhotics Resected (% Resected Cases)	Hospital (H) or Operative (O) Mortality (% Resected Cases)	One Year Survival (%) Resected Cases
Lee et al, 1986[1]	Taiwan	NS	109 (NS)	55 (50.4)	6 (H)	84
Bismuth et al, 1986[2]	France	270	35 (12.9)	35 (100)	15 (O)	66
Kinami et al, 1986[2]	Japan	63	35 (56)	35 (100)	14 (O)	48.6
Li et al, 1985[3]	China	NS	114 (NS)	99 (86.8)	11.4 (O)	58.6
Okuda et al, 1984	Japan	600	98 (16)	NS	NS	62
Lim & Bongard, 1984	USA	86	22 (25.5)	NS	36 (O)	22.7
Okamato et al, 1984	Japan	NS	103 (NS)	NS	12.6 (H)	56.7
Lee et al, 1982	Hong Kong	935	165 (17.6)	NS	20 (H)	45
Okuda et al, 1980	Japan	2411	288 (11.9)	153 (72.3)[4,5]	27.5 (H)[5]	33.35

NS = not stated
1 = includes asymptomatic patients (n = 62)
2 = considers cirrhotic patients only
3 = 14 patients with primary tumours other than HCC included
4 = includes patients with hepatic fibrosis also
5 = figures based on patients with complete follow-up data

Cambridge/King's College Hospital experience has occurred in 38% of their transplanted HCC patients, despite meticulous pre-operative screening for metastases (Polson et al, 1987).

Palliative surgery for HCC is rightly unpopular. Occlusion of the hepatic artery or portal vein may be achieved without recourse to laparotomy (see below).

Emergency surgery may be indicated in those patients who present as an acute abdominal catastrophe due to tumour rupture. Resection should be undertaken if possible, and if it is not, bleeding may be controlled by hepatic artery ligation or by packing the liver. The outlook is poor, but occasional long-term survivors are seen (Ong and Taw, 1972). If the diagnosis is suspected pre-operatively, hepatic angiography may be useful in making the diagnosis and determining resectability, and permits trans-catheter arterial embolisation (TAE) if desired (see below).

OCCLUSION OF THE HEPATIC ARTERY OR PORTAL VEIN

Whereas non-malignant liver receives a dual blood supply from the hepatic artery and portal vein, HCCs are supplied almost exclusively by branches from the hepatic artery (Breedis and Young, 1954). Hepatic artery occlusion may therefore be employed as a treatment for HCC, and is probably best achieved non-operatively by TAE performed at the time of angiography. This technique allows more selective vascular obliteration than is possible at operation and avoids the problems of laparotomy. TAE is hazardous if the portal vein or its

main branches are thrombosed or if liver function is poor (Yamada et al, 1983; Sato et al, 1985a). In these situations portal venous blood flow alone cannot maintain satisfactory liver function. Infarction of non-neoplastic liver may be seen at autopsy in patients dying following TAE in the presence of portal vein thrombosis (Yamada et al, 1983).

Studies of TAE are generally uncontrolled and it remains to be proven convincingly that TAE as sole therapy is of therapeutic benefit in HCC. The results of three recent large Japanese studies are summarised in Table 2 (iii). Revascularisation of tumours is a problem, often necessitating repeated TAE or other forms of therapy.

In patients with ruptured HCC, emergency TAE is an effective and relatively non-invasive method of controlling haemorrhage (Nouchi et al, 1984; Sato et al, 1985b).

Ligation of either the right or left main branch of the portal vein has been advocated as a treatment for HCC (Honjo et al, 1975). This technique is said to be applicable only to non-cirrhotic patients with tumour confined to one liver lobe. Such patients should be suitable for hepatic resection, and palliative portal vein occlusion therefore seems an inappropriate treatment.

Table 2 (iii)

TRANS-CATHETER ARTERIAL EMBOLISATION IN HCC: RESULTS.

AUTHOR	COUNTRY	NO of PATIENTS	SURVIVAL	COMMENTS
Yamada et al, 1983	Japan	120	44% at one year	TAE repeated in 45% of patients. gelatin impregnated with cytotoxic used for embolisation
Furui et al, 1984	Japan	50	51% at one year	TAE repeated in 22% of patients. gelatin impregnated with Mitomycin C used for embolisation
Sato et al, 1985a	Japan	48	median 10.5 months	Survival, if tumour small and vascular, and no evidence of portal vein thrombosis, was better with TAE than with chemotherapy

IRRADIATION

External hepatic irradiation yields disappointing results in HCC treatment (Falkson, 1976; Ong and Chan, 1976; Cochrane et al, 1977). More recently attempts have been made to irradiate hepatic tumours by the administration of radioisotopes bound to anti-tumour antibodies or to resin microspheres, in the hope that such techniques will provide specific high-dose radiotherapy to the tumour.

In a poorly designed uncontrolled study, intravenous ^{131}Iodine bound to polyvalent anti-ferritin antibodies was reported to be of benefit in HCC (Order et al, 1985). Unfortunately the co-administration of cytotoxic agents and external irradiation makes it impossible to evaluate the contribution of the radiolabelled antibody to the observed remission rate of 48% of 105 patients. In a preliminary communication intra-arterially administered ^{131}I-anti-AFP, when given in conjunction with cytotoxic chemotherapy, has been reported to confer some survival advantage over chemotherapy treatment alone (Lin et al, 1983). This result awaits confirmation.

Microspheres containing ^{90}Yttrium have been administered via the hepatic artery to HCC patients (Ariel and Pack, 1970; Grady, 1978). The microspheres become trapped in the tumour vasculature and beta radiation is emitted to a depth of approximately 8 mm. Very few HCC patients have been treated and no conclusion can be reached about the efficacy of such therapy.

CYTOTOXIC CHEMOTHERAPY

The majority of clinical trials of cytotoxic chemotherapy in HCC are small, uncontrolled, and unhelpful. Drugs which show promise in phase II studies require further evaluation in properly conducted phase III trials before their role in HCC therapy can be defined, and such studies are generally lacking. The evaluation of chemotherapy studies would be aided by the universal adoption of a standard set of response criteria, perhaps based on those described by Miller et al (1981).

Single Agent Therapy

The antimetabolite 5-fluorouracil (5-FU) was the first cytotoxic to be extensively investigated as a single agent therapy in HCC. Results were generally poor and the drug is now rarely used. Kennedy et al (1977) reported some benefit from oral 5-FU (six of 12 patients responding) but two other studies (Link et al, 1977; Falkson et al, 1978), the largest of which involved 48 patients, found no responses to oral therapy. Intravenous 5-FU is likewise disappointing (Gailani et al, 1972; Al-Sarraf et al, 1974; Link et al, 1977), though occasional long-term responders may be seen (Davis et al, 1974).

Doxorubicin (Adriamycin) is perhaps the most widely used single agent therapy for inoperable HCC at present. The drug is usually given intravenously every 21 days. A reduced dosage is usually given if jaundice is present, though recent work has suggested that this may be inappropriate and lead to the administration of sub-therapeutic doses (Johnson et al, 1986). Toxicity is significant and side-effects include nausea and vomiting, mucositis, alopecia, bone marrow depression, and dose-related cardiotoxicity. Fear of cardiotoxicity usually limits the total cumulative dose administered to 550 mg/m^2.

The results of studies with doxorubicin are summarised in Table 2 (iv). The initial encouraging results of Olwney et al (1975) have not been observed in subsequent studies (Vogel et al, 1977; Ihde et al, 1977; Johnson et al, 1978c; Olweny et al, 1980; Falkson et al, 1984a; Chlebowski et al, 1984b; Choi et al, 1984; Sciarrino et al, 1985), and response rates are usually less than 20%-30%. Those responding generally do so by the end of the third cycle, and lack of response at this time indicates a need to change therapy.

Anthracyclines which are less toxic than doxorubicin are now available. Mitozantrone is discussed in detail in Chapter 3. Phase II studies so far performed with this agent have produced similar response rates to those observed with doxorubicin but with excellent tolerability (Falkson et al, 1984b; Dunk et al, 1985). 4'epidoxorubicin, a stereoisomer of doxorubicin with reduced cardiotoxicity, has been examined in a small number of HCC patients (Hochster et al, 1985; Shiu et al, 1986). Response rates were disappointing and complications such as nausea and alopecia appeared more common than in the studies with mitozantrone.

A variety of other drugs have been used as single agent therapy for HCC. All have proved disappointing and the results of the best conducted of the many studies are summarised in Table 2 (v). Treatment with interferons is discussed in detail below.

Table 2 (iv)

DOXORUBICIN TREATMENT FOR HEPATOCELLULAR CARCINOMA: RESULTS.

AUTHOR	PATIENTS ENTERED	DOSE	RESPONSE RATE (% OF PATIENTS ENTERED)	SURVIVAL	COMMENTS
Olweny et al, 1975	14	75 mg/m^2 every 21 days	79	median 32 weeks	survival figure based on 11 evaluable patients
Vogel et al, 1977	41	20-75 mg/m^2 every 21 days	20	NS	
Ihde et al, 1977	13	45-75 mg/m^2 every 21 days	15	median 12 weeks	
Johnson et al, 1978c	44	60 mg/m^2 every 21 days	32	median 14 weeks	response criteria not clearly defined
Olweny et al, 1980	74	75 mg/m^2 every 21 days	30	NS	
Falkson et al, 1984a	51	60 mg/m^2 every 21 days for three courses, then every 28 days	12	median 16 weeks	patients given previous chemotherapy excluded from response analysis
Chlebowski et al, 1984b	52	75 mg/m^2 every 21 days	11	0% at one year	only studied patients with serum bilirubin <34 μmol/l
Choi et al, 1984	45	70 mg/m^2 every 21 days	24	median 14 weeks	
Sciarrino et al, 1985	109	60 mg/m^2 every 21 days	10	13% at one year	

NS = Not stated

Table 2 (v)

OTHER SINGLE AGENT THERAPIES EVALUATED IN HCC.

AUTHOR	AGENT	PATIENTS STUDIED	RESPONSE RATE (% TOTAL TREATED)	SURVIVAL	COMMENTS
Ravry et al, 1986	Cisplatinum	29	3	NS	
Melia et al, 1981b	Cisplatinum	13	8	NS	All patients previously treated with other agents. Toxicity severe.
Melia et al, 1983	Etoposide	22	18	NS	In the group randomised to receive Adriamycin, the response rate was similar but of longer duration.
Amrein et al, 1984	Amsacrine	20	5	NS	Patients previously treated by other means
Falkson et al, 1981	Amsacrine	35	3	median 13 weeks	26 patients had static tumour sizes for at least 6 weeks.
Falkson et al, 1984c	Neocarzinostatin	28	7	median 11 weeks	

Multiple Agent Therapy

Cytotoxic drug combinations have little to offer over single agent therapy with doxorubicin. Any marginal benefit in terms of response is invariably offset by unacceptable toxicity. The combination of a variety of agents with doxorubicin, including bleomycin (Ravry et al, 1984), 5-FU (Baker et al, 1977), methyl-CCNU (Chlebowski et al, 1981), methyl-CCNU plus 5-FU (Falkson et al, 1984a), dichloromethotrexate, 5-asacytidine, rezoxane and cyclophosphamide (all Olweny et al, 1980), have all proved disappointing.

Intra-arterial Chemotherapy

There are no satisfactory controlled trials comparing the intra-arterial and intravenous routes of drug administration. The uncontrolled and generally unimpressive results of intra-arterial chemotherapy for HCC have recently been reviewed (Opfell and Bowen, 1985). Any benefit of intra-arterial over intraveous therapy in terms of response is probably outweighed by the technical problems of intra-arterial treatment (Bottino, 1985), though these are becoming less common as new infusion devices are developed. The recent suggestion that intra-arterial doxorubicin may precipitate hepatic failure in HCC patients with cirrhosis is worrying and requires clarification (Tommasini et al, 1986).

The oily X-ray contrast medium Lipiodol is selectively retained in tumour tissue for many months after intra-arterial administration, and CT scanning seven-10 days after intra-arterial Lipiodol has been suggested as superior to either CT scanning or angiography alone in detecting the true extent of HCC spread within the liver (Yumoto et

al, 1985). Lipophilic cytotoxic drugs such as styrene maleic acid neocarzinostatin (SMANCS) have been solubilised in Lipiodol and administered intra-arterially to HCC patients in the hope of prolonged retention of cytotoxic activity within the tumour (Tashiro and Maeda, 1985). An uncontrolled Japanese study of 94 patients, in which response criteria were not accurately defined, has yielded encouraging results which require confirmation in a properly controlled and blinded trial (Tashiro and Maeda, 1985). Hydrophilic drugs such as doxorubicin may be successfully admixed with Lipiodol in the presence of the X-ray contrast material Urografin, which is water soluble but of the same specific gravity as Lipiodol (Kanematsu et al, 1984). The results of an uncontrolled study of this form of therapy are of encouragement (Kanematsu et al, 1984). The use of "lipiodolised" cytotoxic drug therapy merits full evaluation in controlled studies with "conventional" intravenous and intra-arterial treatment.

IMMUNOTHERAPY

Levamisole, a stimulator of cell-mediated immunity, did not improve the survival of Oriental patients when given after recovery from operative hepatic arterial obliteration (Plengvanit et al, 1986).

Interferons possess a wide range of immunomodulatory and direct antiproliferative properties (Gresser and Tovey, 1978; Borden and Ball, 1981; Steihm et al, 1982), and because of these, they are now being extensively examined as potential therapies for a variety of malignancies. Three studies in small numbers of HCC patients have so far been reported. Sachs et al (1985) found recombinant leucocyte interferon to be of no benefit to 16 black South African patients, and

toxicity was such that in 10 patients the drug had to be either discontinued or the dosage reduced. A lower dose of a leucocyte interferon preparation was found by Nair et al (1985) to be safe and capable of immune stimulation, though no responses to therapy were noted in their study of only five patients. Recombinant gamma interferon in a dose of 2.7×10^6 IU/m^2 produced no responses to therapy after four weeks treatment and toxicity was such that the study was discontinued after recruitment of only seven patients (Forbes et al, 1985). These trials allow one to conclude only that interferons possess significant toxicity when given to HCC patients in doses that would usually be tolerable to subjects without severe parenchymal liver disease. The numbers of patients studied are too small to allow meaningful assessment of anti-tumour activity. Further studies are needed to determine the toxicity and efficacy of low dose intereron therapy, and the possible synergy which exists between interferons and the anthracyclines (Green et al, 1985) may be worthy of exploration in HCC.

Immunotherapy with antibodies directed against HCC-associated antigens is an exciting prospect for future treatment, and may enable the selective targeting of drugs, isotopes or potent cellular toxins to tumour cells. Clinical trials in a variety of different malignancies are now under way and these have recently been reviewed by Morgan and Foon (1986) and Baldwin and Byers (1986). The uncontrolled studies so far performed in HCC patients (Liu et al, 1983; Order et al, 1985), both involving ^{131}I- labelled polyvalent antibodies have already been discussed. As yet there are no reported studies of HCC immunotherapy with monoclonal antibodies to non-secreted cell surface antigens, and

no reports of HCC therapy with antibodies conjugated with either cytotoxic drugs or toxins.

Recently, lymphokine activated killer (LAK) cells have been suggested as a potential form of passive ("adoptive") immunotherapy in cancer patients. Preliminary studies in which LAK cells and interleukin-2 (IL-2) have been infused together demonstrated remission in 11 of 25 patients with a variety of advanced malignancies (Rosenberg et al, 1985). In vitro experiments in a small number of HCC patients have shown that their IL-2 stimulated killer cells have enhanced cytotoxicity against fresh autologous and allogeneic tumour cell suspensions and established HCC cell lines (Hsieh et al, 1987). So far only one HCC patient has received intra-arterial treatment with autologous LAK cells (Okuno et al, 1986). A transient fall in AFP was noted, with levels soon rising to pre-treatment values again. IL-2 was not infused concurrently.

HORMONAL THERAPY

The rationale behind the suggestion that HCC may be a hormonally dependent tumour is discussed in Chapter 5. Two small uncontrolled studies have shown modest objective responses of HCC to hormonal manipulation with cyproterone acetate (Forbes and Williams, 1987) and a combination of tamoxifen and norethisterone (Trinchet et al, 1985). There is also anecdotal evidence of HCC regression in response to progestogen therapy (Friedman et al, 1982). Clinical trials with tamoxifen are currently underway in South Africa, though no benefit has so far been seen with this agent when given alone (Paliard et al, 1984) or in combination with cytotoxic drugs (Melia et al, in press).

COMBINATION THERAPIES

No satisfactory controlled clinical trials of combinations of different modalities in HCC treatment exist. As any advantage of combination therapy over treatment with one single modality is likely to be small, large numbers of patients are required for such trials, in order that small treatment differences may be detected with confidence.

CONCLUSIONS

HCC remains a disease of grave prognosis, mainly because the tumour usually presents late in its natural history, when operation is not possible. The results of operation in early disease are of some encouragement and every effort should be made to regularly screen the cirrhotic population at risk for HCC development.

The outlook for inoperable patients remains gloomy though exciting new treatment modalities exist which require extensive evaluation. An anthracycline given as single agent intravenous therapy is probably the current treatment of choice for these patients, though only 20%-30% will show a response.

CHAPTER THREE

MITOZANTRONE AS SINGLE AGENT THERAPY IN HEPATOCELLULAR CARCINOMA

ABSTRACT

Thirty-five patients with HCC have been treated with the Anthracenedione mitozantrone (dihydroxy-anthracenedione dihydrochloride). One complete and five partial responses to therapy were seen, and in a further four patients tumour size remained static for lengths of time ranging from 13 to 42 weeks.

Therapy was well tolerated. Bone marrow suppression was dose-limiting but easily managed by dose reduction. Other acute toxicity was rare, vomiting and hair loss being reported only once each. Cardiac 'events' occurred in five patients, three of whom had received high total cumulative doses of mitozantrone.

It is concluded that mitozantrone is of clinical benefit in some patients with HCC and that its usage is compatible with a good quality of survival. Its cardiotoxic potential requires further evaluation.

INTRODUCTION

As discussed in Chapter 2, cytotoxic chemotherapy is currently the mainstay of treatment in HCC, though results are generally disappointing. Doxorubicin (Adriamycin) is usually regarded as the drug of choice, though response rates are generally less than 20%-30% (Vogel et al, 1977; Ihde et al, 1977; Johnson et al, 1978c; Olweny et al, 1980; Falkson et al 1984a; Chlebowski et al, 1984b; Choi et al, 1984; Sciarrino et al, 1985). Doxorubicin is toxic and frequently causes nausea, vomiting, alopecia and mucositis. A serious

disadvantage is dose-dependent cardiotoxicity (Lefrak et al, 1973; Unverferth et al, 1982) which may limit its continued useage in those patients who show a useful clinical response. New chemotherapeutic agents are urgently needed which are both less toxic and more efficacious in HCC than doxorubicin.

Mitozantrone (Figure 3(i)) is an anthracenedione structurally related to doxorubicin but without the amino-sugar moiety postulated by Adamson (1974) to be responsible for doxorubicin cardiotoxicity. In animal model systems mitozantrone has shown anti-tumour activity comparable and often superior to doxorubicin, in association with minimal or absent cardiotoxicity (Fujimoto and Ogawa, 1982; Henderson et al, 1982; Schabel et al, 1983). Phase I studies have suggested that mitozantrone is well tolerated and has a low incidence of serious side-effects (Von Hoff et al, 1980; Alberts, 1980), and early phase II studies suggest that mitozantrone may be of benefit in breast cancer, acute leukaemia and the malignant lymphomas (Prentice et al, 1983; Coltman et al, 1983; Stuart-Harris et al, 1984). In this chapter the findings of a multicentre phase II study of mitozantrone as single agent therapy in inoperable HCC are reported.

PATIENTS AND METHODS

Patients were considered eligible for study if they had histologically proven HCC or a serum AFP > 500 IU/ml in conjunction with a hepatic arteriogram showing hypervascular tumour circulation. Patients were excluded from the study if they had received any prior form of therapy for HCC, if the tumour was considered resectable, or if they were of WHO performance grade 4 (World Health Organisation, 1979), i.e.

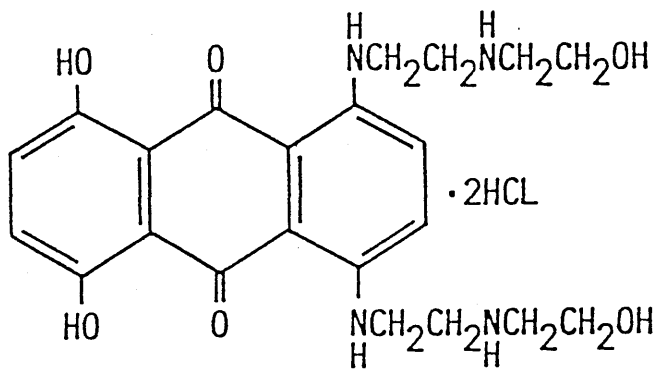

Figure 3 (i)

CHEMICAL STRUCTURE OF MITOZANTRONE.

completely disabled and totally confined to bed or chair. Between January 1982 and January 1984, 35 suitable patients were enrolled for study. A further five subjects who had previously had some other form of therapy for HCC were subsequently given mitozantrone as single agent therapy. They have been considered only for toxicity analysis.

The characteristics of the 35 patients considered suitable for study are shown in Table 3(i). There were 29 men and six women. Ages ranged from 10 years to 76 years with a mean age of 56.8 years (median 57 years). Twenty-one patients were non-British. Thirty patients had disease confined to the liver alone and five patients had metastatic HCC. Three patients (numbers 2, 9 and 14) had multiple pulmonary metastases, one patient (number 4) had bony metastases and one patient (number 7) had HCC deposits in supraclavicular lymph nodes. HBsAg was detected in the serum by radioimmunoassay (Ausria, Abbot Laboratories, UK) in eight patients. Cirrhosis was present in 24 patients and absent in seven, and the state of the underlying non-malignant liver in the remaining four patients was not known. The diagnosis of HCC was confirmed histologically in 25 subjects and in the remainder was based on a serum AFP of > 500 IU/ml (RIA-gnost AFP, Hoescht Laboratories, UK) and a characteristic angiogram. Serum AFP was < 10 IU/ml in five subjects, between 10 and 100 IU/ml in three, between 101 and 500 IU/ml in four and >500 IU/ml in the remaining 23 subjects.

Prior to treatment all patients were fully examined and the size of the enlarged liver was recorded by measuring in the mid-clavicular line the distance between the right costal margin and the liver edge at the end of quiet inspiration. Liver function tests, full blood count and serum AFP were measured immediately before treatment and an

Table 3 (i)

PRETREATMENT PATIENT CHARACTERISTICS AND RESPONSES TO THERAPY.

Patient No	Sex/ Age (yr)	Race	HBsAg	AFP (IU/ml)	Cirrhosis	WHO Performance Grade	Response	Duration of Response (weeks)
1	M/52	Greek	-	4000	-	1	PD	-
2	F/53	British	-	<10	-	2	NE	-
3	M/52	Saudi	+	360000	-	3	PD	-
4	F/55	British	-	11520	-	0	PD	-
5	M/70	Saudi	-	600	NK	2	NE	-
6	M/35	Saudi	+	13	-	0	PD	-
7	M/21	British	-	<10	-	0	PD	-
8	M/55	Saudi	-	48800	NK	1	PD	-
9	M/52	Greek	-	6900	+	1	NE	-
10	M/71	British	-	26400	+	1	NE	-
11	M/63	British	-	133	+	0	PD	-
12	F/63	British	-	<10	-	0	PD	-
13	M/75	Omani	+	147	+	3	PD	-
14	M/52	British	-	91200	+	1	NE	-
15	M/57	Quatari	-	13900	+	3	NE	-
16	M/66	British	+	13100	+	3	NE	-
17	M/46	Nigerian	-	4000	+	1	PD	-
18	M/59	British	-	16000	+	3	NE	-
19	M/59	West Indian	-	125	+	3	NE	-
20	M/56	Zambian	+	23500	NK	2	NE	-
21	M/56	Saudi	+	58	-	2	NE	-
22	M/76	British	-	43800	+	2	NE	-
23	M/73	British	-	6200	+	1	PD	-
24	M/69	Saudi	-	7330	+	1	NE	-
25	M/66	French	-	8730	+	1	PD	-
26	M/50	Italian	-	37200	+	1	CR	13
27	M/69	British	-	1769	+	1	PR	3
28	M/65	Omani	-	3041	+	2	PR	6
29	M/53	Italian	-	1662	+	1	PR	6
30	M/10	Israeli	-	37700	-	0	PR	17
31	M/52	Italian	-	62	+	1	PR	29
32	F/62	British	-	<10	-	0	NC	24
33	F/61	Yugoslavian	-	2100	+	1	NC	29
34	F/76	British	-	<10	NK	1	NC	42
35	M/38	Omani	+	318	+	2	NC	13

Abbreviations: NK = Not Known; NE = Not Evaluable; PD = Progressive Disease;
CR = Complete Response; PR = Partial Response; NC = No Change

electrocardiogram (ECG) performed. Pretreatment tumour size was also assessed by computerised tomography of the liver or by ultrasound examination. Mitozantrone was diluted in 5% dextrose and 12 mg/m^2 was infused intravenously over 10-30 minutes every 21 days. The dosage was increased or decreased by increments of 2 mg/m^2 depending on toxicity and response. In mid-cycle (between days 10 and 14) the full blood count was measured and immediately prior to each subsequent cycle of therapy this was repeated, as were the liver function tests and the serum AFP. The patients' WHO performance grade and adverse events following each cycle were recorded using a standard questionnaire with toxicity for each body system graded 0-4 according to the WHO classification (World Health Organisaton, 1979). Tumour size was assessed clinically during every cycle and CT or ultrasound examination were repeated after every two to three cycles.

Response criteria were defined using a modification of internationally accepted criteria (Miller et al, 1981). A complete response (CR) was defined as complete disappearance of all known tumour deposits and a decrease in the serum AFP to less than 20 IU/ml in those patients in whom this parameter was initially elevated. A partial response (PR) required a reduction in the total size of all measurable lesions of at least 50%, or a greater than 50% fall in serum AFP concentration. Where the liver was diffusely replaced by tumour, response was assessed using clinical measures of liver size and a PR required at least a 50% reduction in hepatomegaly as measured by distance below the right costal margin in the mid-clavicular line. A response was considered to have occurred if the required change occurred in one parameter, provided there was no contradictory change in the other

parameter. "No change" (NC) was defined as a change in total tumour size or AFP concentration which amounted to less than a 50% reduction or a 25% increase in either parameter. Progressive disease (PD) was defined as the appearance of any new lesion or a greater than 25% increase in either tumour size or serum AFP concentration. Patients were considered non-evaluable for response analysis if they did not complete six weeks of therapy or if there were insufficient follow-up data, or if a major protocol violation affecting treatment scheduling occurred.

RESULTS

Responses to treatment

Of the 35 patients eligible for study, 22 were evaluable for response and 13 were not. The reasons for non-evaluability are listed in Table 3 (ii). The main reason was early death due to malignancy, defined as death attributable to malignancy occurring within six weeks of starting treatment. A complete response was seen in one patient (4.5% of those evaluable) and lasted 13 weeks. There were five partial responses (22.7%) of mean duration 12 weeks (range 3-27 weeks). Four patients (18.2%) remained in the "no change" category for a mean duration of 27 weeks (range 13-42 weeks) and the remaining 12 evaluable patients (54.6%) showed evidence of progressive disease.

Patients who showed a complete or partial response to therapy responded within the first six weeks of treatment. In those patients in whom it was raised, serum AFP fell rapidly and CT scan or ultrasound showed a > 50% reduction in tumour size. In all patients responding, general well-being, measured in terms of WHO performance

Table 3 (ii)

NON-EVALUABLE PATIENTS: REASONS.

Patient numbers	Reasons
2, 24	Protocol violation
9	Inadequate data
5, 10, 14-16, 18-22	Early death due to malignancy*

* death < 6 weeks from start of treatment

grade, either remained static (four patients) or improved (two patients). Patients in the "no change" category showed static (two patients) or improved (two patients) performance grade, and tumour size and serum AFP concentration remained around pretreatment values. In contrast, patients with progressive disease refractory to mitozantrone showed a deterioration in performance grade and a steadily increasing tumour size was noted by CT scan or ultrasound examination.

Mitozantrone therapy was continued in all groups of patients until they showed evidence of progressive disease (a > 25% increase in the lowest recorded tumour size or AFP concentration). Therapy was then changed to a variety of other agents and all patients were followed up until death.

The median duration of survival of patients with progressive disease was significantly shorter than that for those who showed "no change" or some form of response to treatment (17 weeks vs 82 weeks, $p<0.05$, log-rank test). The median duration of survival of the 22 evaluated patients was 53 weeks, and that for the 35 patients originally eligible for study was 16 weeks (Figure 3 (ii)).

Toxicity

Toxicity analysis was performed on data collected from the 35 patients entered into the Phase II study and from a further five patients who had previously had other forms of treatment for HCC.

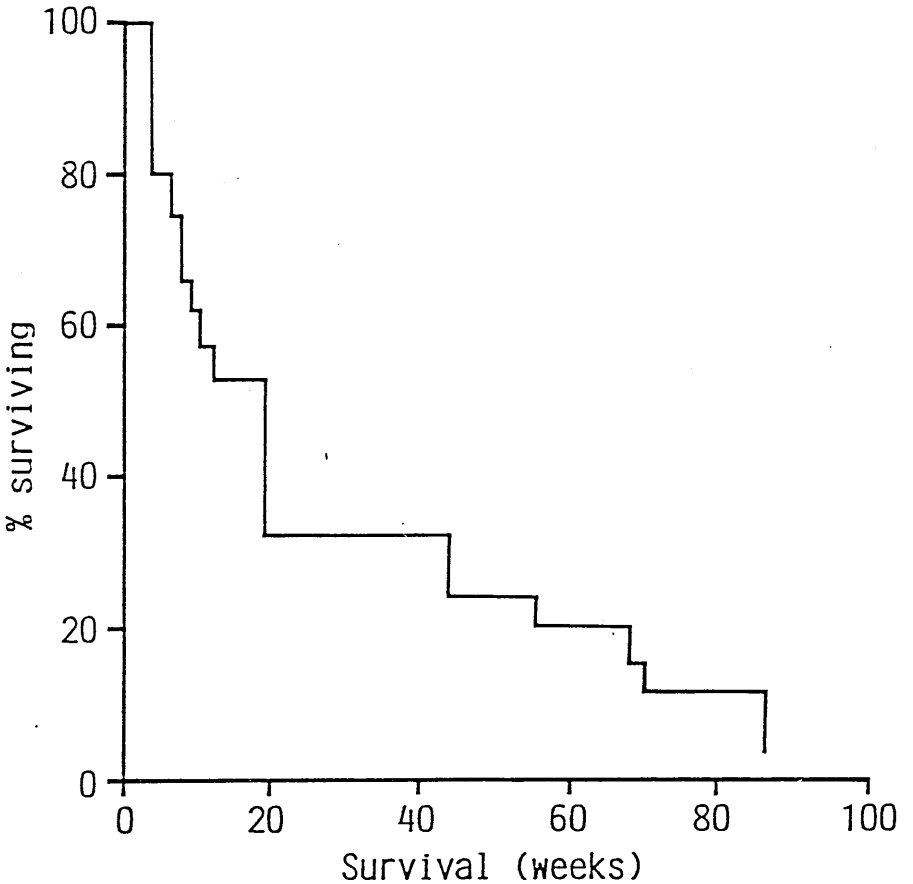

Figure 3 (ii)

KAPLAN-MEIER SURVIVAL CURVE OF ALL PATIENTS (n = 35) ELIGIBLE FOR STUDY.

Changes in total white cell and platelet counts are illustrated in Figure 3 (iii). A total white cell count (WCC) of less than 1.0 x 10^9/l was noted on four occasions and two patients developed infections during treatment. One patient developed septicaemia which responded to appropriate treatment during a cycle associated with a nadir WCC of 1.1 x 10^9/l and another developed a urinary infection associated with renal calculi and a nadir WCC of 0.4 x 10^9/l. A platelet count of less than 40 x 10^9/l occurred on four occasions and one patient with renal calculi (the same patient as that with the urinary infection) developed haematuria associated with a nadir platelet count of 66 x 10^9/l.

Cardiac toxicity was detected clinically and by ECG, which was performed after every second or third cycle. In addition, six patients had radionuclide ventriculography prior to mitozantrone therapy, which was then repeated after every third cycle of therapy. This is an accepted non-invasive technique for the assessment of left ventricular function, and testing was performed both at rest and on stress (Alexander et al, 1979). Cardiac events were noted in five of 40 patients (12.5%) and are listed in Table 3 (iii). One patient with pre-existing left bundle branch block developed atrial fibrillation 24 hours after the first injection of mitozantrone. This responded to digitalis treatment and did not recur on subsequent injections. A further patient, who had been heavily pre-treated with doxorubicin and who had developed incomplete right bundle branch block while on this drug, developed complete heart block 48 hours after the first injection of mitozantrone. The patient died six days later, still in complete heart block. Cardiac events occurred in three other

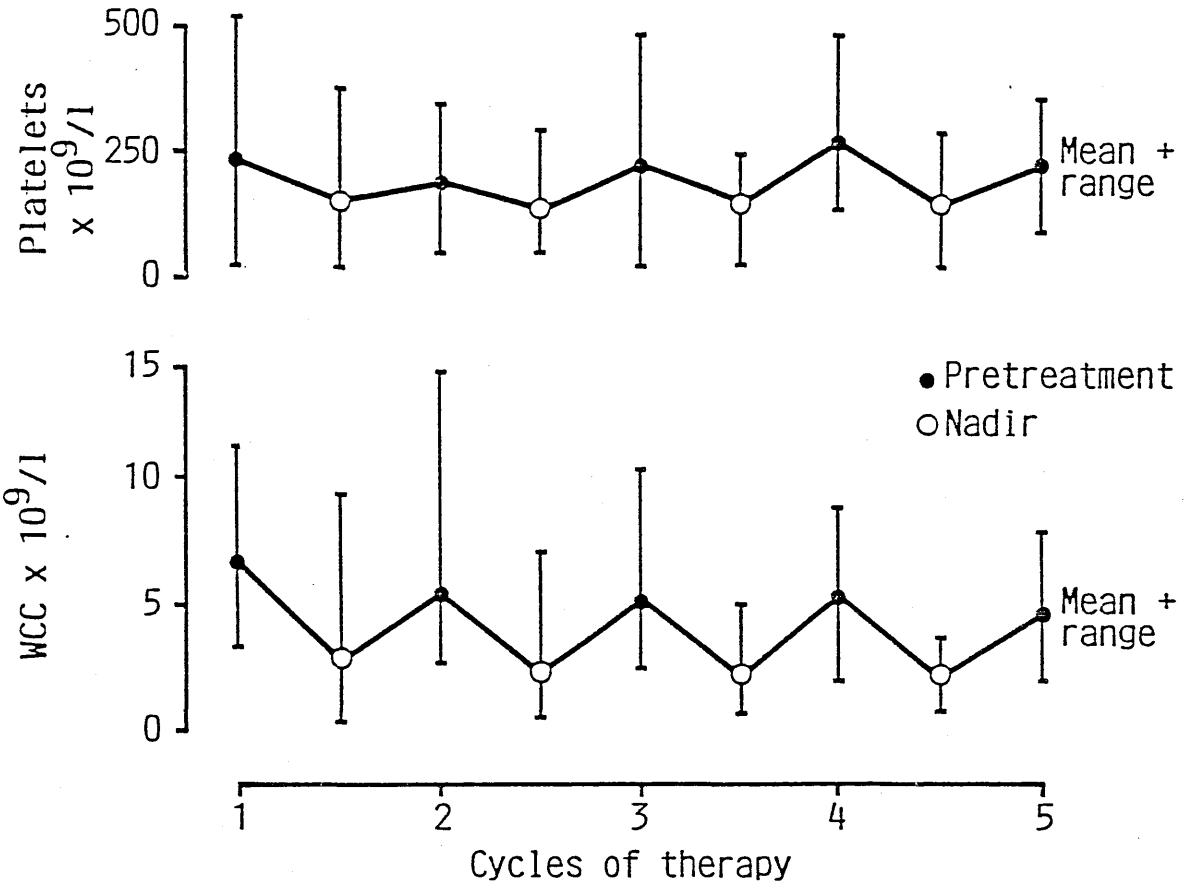

Figure 3 (iii)

PRETREATMENT AND NADIR WHITE CELL AND PLATELET COUNTS DURING MITOZANTRONE THERAPY.

Table 3 (iii)

CARDIAC EVENTS DURING MITOZANTRONE TREATMENT.

Patient number	Age (yr)	Event	Cumulative mitozantrone dose (mg/m^2) at event	Risk factors
29	53	Atrial fibrillation	12	LBBB
-	48	Complete heart block	12	Doxorubicin 480 mg/m^2 incomplete RBBB
34	76	Reduced LV function	132	--
33	61	PAT	134	--
4	55	LBBB	126	--

Abbreviations: LBBB = left bundle branch block; RBBB = right bundle branch block; LV = left ventricular; PAT = paroxysmal atrial tachycardia.

patients, none of whom had any risk factors for cardiac disease. All had received high total cumulative doses of mitozantrone (Table 3 (iii)). Mitozantrone was discontinued and cardiac function did not deteriorate further.

Other treatment-related side-effects are listed in Table 3 (iv). The drug was generally well tolerated. Only one patient experienced vomiting during mitozantrone treatment and this was limited to the day of injection. Hair loss, an almost universal problem with doxorubicin, was reported by only one patient and was not cosmetically troublesome. The most frequent side-effect was WHO grade 1 nausea whch was experienced by 27% of patients.

DISCUSSION

As discussed in Chapter 2, doxorubicin is widely accepted as the best single agent form of therapy currently available for inoperable HCC. Response rates however are poor, being generally less than 20-30% (see Table 2 (iv), p47), and the combination of other cytotoxic agents with doxorubicin has so far either failed to improve these depressing results (Baker et al, 1977; Chlebowski et al, 1981), or has marginally done so at the expense of severe drug-related toxicity (Falkson et al, 1984a).

In this study, one complete and five partial responses were noted in 22 evaluable patients, an overall response rate of 27.3%. In addition, four further patients remained in the "no change" category for clinically significant lengths of time ranging from 13-42 weeks.

Table 3(iv)

OTHER SIDE EFFECTS SEEN DURING MITOZANTRONE THERAPY.

Side-effect	WHO grade of severity*					Patients with insufficient toxicity data	Total number of patients
	0	1	2	3	4		
Nausea/vomiting	26	9	1	0	0	4	40
Fever	31	4	1	0	0	4	40
Hair loss	35	1	0	0	0	4	40
Cutaneous reactions	35	0	1	0	0	4	40
Injection site reactions	35	1	0	0	0	4	40
Diarrhoea	34	1	1	0	0	4	40
Other	34	1	1	0	0	4	40

* Roughly, grade 0 means side-effect absent. Grades 1 and 2 are equivalent to 'mild' side-effects requiring no treatment, and grades 3 and 4 side-effects require treatment.

Thus we feel that mitozantrone was of clinical benefit in 45.4% of our evaluable patients (28.6% of the 35 patients originally entered). The only other phase II study of mitozantrone in HCC (Falkson et al, 1984b) has been performed in a small number of predominantly black patients from South Africa and a response rate of only two from 17 patients (11.7%) was noted, a figure similar to that observed in many of the studies with doxorubicin. Clearly a phase III study comparing mitozantrone and doxorubicin in HCC is indicated.

Mitozantrone produced a statistically significant increase in survival in those patients who had a response to treatment or remained in the "no change" category, when compared to those patients with progressive disease refractory to mitozantrone. It should be noted however that the median duration of survival of the whole group of patients originally considered eligible for study was only 16 weeks. This disappointing figure is accounted for by the large number of early deaths due to malignancy seen in those patients of WHO performance grades 2 and 3 (eight of the 10 early deaths due to malignancy seen), and our experience would suggest that these patients should probably not be included in phase II studies of cytotoxic agents in HCC, as they are unlikely to survive long enough to be evaluable for response.

Mitozantrone treatment was given on an out-patient basis once every 21 days. A standard dosage regime was used in all patients irrespective of liver function, and was well tolerated.

Myelosuppression was rarely a clinical problem and could easily be managed by dosage adjustment. There was no evidence of significant cumulative myelosuppression with continued mitozantrone usage.

Doxorubicin causes both acute and chronic cardiac toxicity (Lefrak et al, 1972; Unverferth et al, 1982). The latter dose-dependent cardiotoxicity may lead to congestive cardiac failure and death and becomes increasingly common at cumulative doses of over 540 mg/m^2. Mitozantrone, in animal model systems, has shown little or no cardiotoxic potential (Henderson et al, 1982; Fujimoto and Ogawa, 1982; Schabel et al, 1983). In this study five cardiac events were noted. Two subjects developed serious cardiac arrythmias within 48 hours of their first injection of mitozantrone. Both had pre-existing ECG abnormalities and one had been heavily pre-treated with doxorubicin. The other three cardiac events occurred at high cumulative doses of mitozantrone, in subjects with no pre-existing cardiac disease. Mitozantrone therapy was withdrawn and no further deterioration in cardiac function was noted in these patients. Cardiotoxicity has been noted during mitozantrone treatment by several other investigators (Schell et al, 1982; Unverferth et al, 1983) and prior treatment with anthracyclines seems to increase the risk of its development. Until the cardiotoxicity of mitozantrone is fully evaluated it is currently recommended that treatment proceed with caution above cumulative dose levels of 100-120 mg/m^2 in anthracycline pre-treated patients and above 140-160 mg/m^2 in non-pretreated patients (Crossley, 1983).

Other side-effects were uncommon and mild, and the quality of survival of responding patients was excellent. Only one patient suffered vomiting and only one patient reported hair loss during treatment. This was of WHO grade 1 severity only.

We conclude that mitozantrone appears to have activity in HCC, probably at least equal to that seen with other cytotoxic agents, and that its usage is associated with a low incidence of serious side-effects, though its potential cardiotoxicity requires further evaluation. It should be noted however, that the median duration of survival of our 35 patients was only 16 weeks from the start of treatment. New methods are therefore needed for diagnosing HCC at an early stage, when surgical cure might be possible, and novel modes of treatment such as interferon therapy (Chapters 5 and 6), hormonal manipulation (Chapter 7), and immunotherapy with anti-HCC monoclonal antibodies (Chapter 8), should be urgently evaluated.

CHAPTER FOUR

HUMAN HEPATOCELLULAR CARCINOMA CELL LINES AND THE PRODUCTION OF TUMOUR XENOGRAFTS IN ATHYMIC (NUDE) MICE: BACKGROUND AND METHODOLOGY

INTRODUCTION

New and more efficacious cytotoxic drugs are urgently required for HCC treatment, and novel modes of treatment such as interferon therapy (Chapters 5 and 6), hormonal manipulation (Chapter 7) and immunotherapy with anti-HCC monoclonal antibodies (Chapter 8) require evaluation. Many of the experiments detailed in subsequent chapters have utilised human HCC tumour xenografts growing in athymic (nude) mice as an animal model for the preliminary testing of the *in vivo* response of human HCC to such therapies and as a ready source of tumour tissue for *in vitro* study. This chapter briefly describes the characteristics of the athymic mouse and its role in the screening of new anti-cancer therapies, and also details the materials and methods used for the *in vitro* culture of human HCC cell lines, the housing of athymic mice, and the production of HCC tumour xenografts.

ATHYMIC (NUDE) MICE: THEIR HISTORY AND ROLE IN CANCER RESEARCH

Flanagan (1961) was the first to describe the nude mouse mutant, so called because of the almost total absence of body hair in homozygotes. He recognised the autosomal recessive mode of inheritance of the trait and that when kept under conventional conditions, these animals had a short life span. It was Pantelouris (1968), working at Strathclyde University, who first reported thymic dysgenesis in nude mice, and shortly thereafter, in the light of the then recent discovery of thymus-dependent cell-mediated immunity (Miller, 1961), the first reports of the transplantation of human tumours in athymic mice and the production in these immunologically incompetent animals of tumour xenografts derived from tumour cell lines were published (Rygaard and Povlsen, 1969; Giovanella et al,

1972). Since that time the immunological status of athymic mice has been extensively investigated (Kindred, 1978; Herberman, 1978; Giovanella and Fogh, 1985), many tumours have been grown as xenografts (Sharkey et al, 1978), and the animal is now widely used as an *in vivo* test system for screening the cytotoxic activity of new anti-cancer therapies. The advantages of the athymic mouse over other *in vivo* systems which may be used for this purpose have recently been reviewed (Giovanella and Fogh, 1985), as have the limitations of the nude mouse tumour xenograft model when used to screen anti-cancer drug activity (Sharkey and Fogh, 1984; Giovanella and Fogh, 1985). An obvious disadvantage, as discussed in more detail in Chapter 5, is the inability to accurately assess the effects on tumour growth of agents which act partly or wholly on the host immune system.

Bassendine et al (1980), using the PLC/PRF/5 cell line (see below), described the production of HCC xenografts in athymic mice. In these studies the morphology of xenografted tumours was similar to that of a well differentiated HCC, and xenografts produced HBsAg and AFP in amounts readily detected in mouse serum. Subsequent studies (Bassendine et al, 1983) determined the growth charactersitics of PLC/PRF/5-derived xenografts and revealed a strong positive correlation between tumour xenograft mass and mouse serum AFP concentration. The ease with which the PLC/PRF/5 cell line can be xenografted, and the ability to utilise tumour size, serum AFP concentration, and mouse survival as indicators of reponse to therapy, make the athymic mouse-tumour xenograft model the best currently available *in vivo* test system for the rapid preliminary evaluation of potential new anti-HCC therapies.

HCC CELL LINES

In almost all of the experiments to be detailed subsequently the PLC/PRF/5 cell line was chosen as the human HCC cell line for study. This cell line was established in 1973 and is derived from the tumour of a 24 year old Mozambican male whose serum was positive for both HBsAg and AFP (Alexander et al, 1976). PLC/PRF/5 cells contain genomically integrated HBV-DNA sequences and secrete HBsAg but no other HBV proteins (Alexander et al, 1976; Bassendine et al, 1980; Chakraborty et al, 1980). AFP, though not detectable when PLC/PRF/5 cells are grown in tissue culture, is readily detectable when cells are grown as xenografts (Bassendine et al, 1980).

The SK-Hep-1 and Mahlavu cell lines, which are utilised in Chapter 7, are poorly described in the literature. The SK-Hep-1 cell line is derived from the tumour of a male patient (Fogh and Trempe, 1975) and does not produce HBsAg (Shouval et al, 1983). The sex of the patient from whom the Mahlavu cell line is derived is not stated in the literature (Prozesky et al, 1973). This cell line contains integrated HBV-DNA sequences in low copy number and produces HBsAg only when stimulated by dexamethasone (Oefinger et al, 1981).

CELL CULTURE CONDITIONS AND THE PRODUCTION OF HCC TUMOUR XENOGRAFTS

PLC/PRF/5 cells were grown as monolayers in modified Eagle's medium (MEM) with Earle's salts (Gibco Ltd, Paisley, UK), and the SK-Hep-1 and Mahlavu cell lines were both grown as monolayers in RPMI 1640 medium (Flow Laboratories, Irvine, UK). Both media were supplemented with 10% v/v foetal calf serum, L-glutamine and antibiotics (Gibco Ltd, Paisley, UK). Cells were grown at 37^{o}C in a humidified

atmosphere of 95% air and 5% CO_2, and media were changed twice weekly. Cells were harvested by mild trypsinisation and gentle agitation in trypsin - EDTA.

All animal studies utilised sexually mature male athymic (nu/nu) mice of either BALB/c or CBA strain. Each experiment involved mice of only one strain. Athymic mice were housed in sterile filter cages in a room separate from other experimental animals. Laboratory feed was irradiated and drinking water was acidified to minimise the risk of pseudomonas infection. All handling was performed using strict aseptic techniques.

Tumour xenografts were produced as described by Bassendine et al (1980). Mice received sub-lethal whole body irradiation of 450 rads. Forty-eight hours later 10^7 viable tumour cells, as determined by trypan blue dye exclusion, were injected subcutaneously into the loose tissues over the neck region. Tumour xenografts usually appeared within three to six weeks of injection. Xenografts of each of the three HCC cell lines grew as localised, well circumscribed, nodular tumours (Figure 4(i)). No evidence of local invasion of deeper structures or of distant metastases was found at post-mortem, which was performed on almost all animals studied.

Only PLC/PRF/5-derived xenografts, whose growth characteristics in athymic mice have been detailed previously (Bassendine et al, 1983), were used in experiments studying tumour growth <u>in vivo</u>. Tumour growth was assessed both by measuring the two largest tumour diameters at right angles to each other with precision calipers and obtaining the product, the tumour base area, and by measuring mouse serum AFP

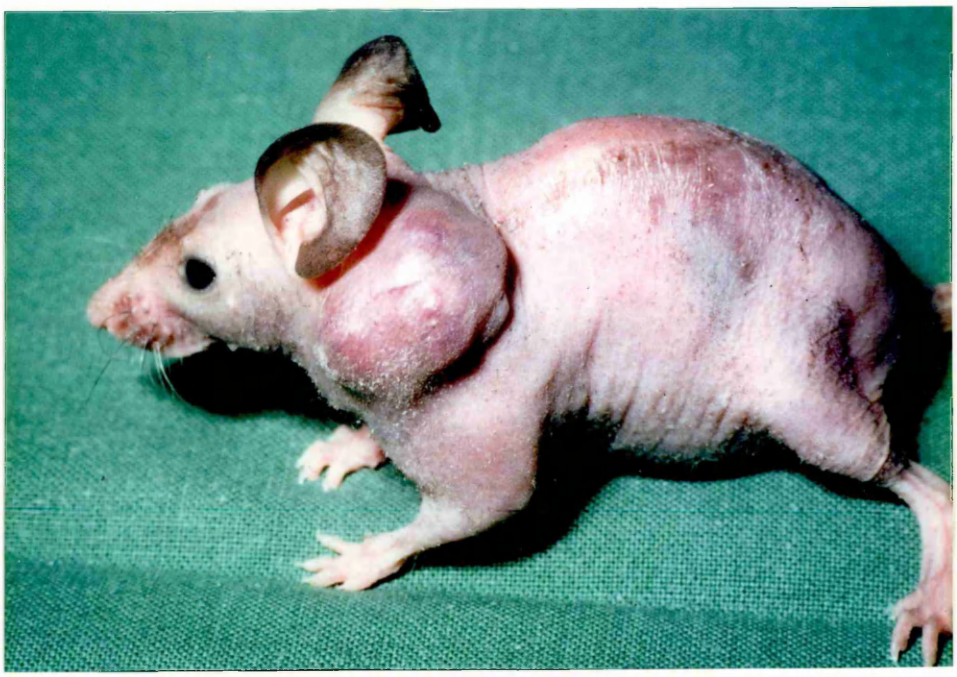

Figure 4 (i)

ATHYMIC (NUDE) MOUSE WITH A TUMOUR XENOGRAFT DERIVED FROM THE PLC/PRF/5 CELL LINE.

concentration by radioimmunoassay (RIA-gnost AFP, Hoechst Laboratories, UK). Animals with PLC/PRF/5-derived tumour xenografts survive approximately four to eight weeks from the time of the tumour development (Bassendine et al, 1980).

CHAPTER FIVE

THE EFFECTS OF HUMAN LYMPHOBLASTOID INTERFERON ON THE GROWTH OF THE HUMAN HEPATOCELLULAR CARCINOMA CELL LINE PLC/PRF/5: STUDIES IN VITRO AND IN ATHYMIC MICE WITH PLC/PRF/5-DERIVED TUMOUR XENOGRAFTS

ABSTRACT

The growth inhibiting effects of human lymphoblastoid interferon (Hu IFN-α[Ly], IFN) on the human HCC cell line PLC/PRF/5 have been examined. *In vitro*, PLC/PRF/5 cells were sensitive to the antiproliferative effects of IFN, growth inhibition being noted at concentrations as low as 1.25 IU/ml. Athymic mice with PLC/PRF/5-derived tumour xenografts were treated daily with IFN or a saline control. An IFN dose of 2×10^5 IU/day was found capable of significantly reducing tumour growth rate and prolonging mouse survival. Further studies to examine the mechanisms involved in growth inhibition *in vivo* demonstrated that IFN was capable of inducing the activity of the enzyme 2,5-oligoadenylic acid (2,5 A) synthetase, a potent inhibitor of protein synthesis, in tumour xenografts but not in mouse tissue, and that IFN significantly enhanced the membrane display of HLA class I glycoproteins on tumour cells, though histology did not reveal any increase in tumour infiltration by host lymphocytes. It is concluded that IFN exerts potent growth inhibiting effects on the PLC/PRF/5 cell line both *in vitro* and *in vivo* and its mode of action in the athymic mouse system appears to be predominantly mediated by a direct antiproliferative effect on tumour cells.

INTRODUCTION

The interferons are a family of proteins and glycoproteins which were first described as naturally occurring anti-viral agents (Isaacs and Lindenmann, 1957) but which have since been shown to possess a variety of immunoregulatory and antiproliferative properties (Gresser and Tovey, 1978; Borden and Ball, 1981; Steihm et al, 1982). It is

because of these latter properties, and because of increasing availability brought about predominantly by advances in recombinant DNA technology, that the interferons are now the focus of much attention as potential anti-cancer agents, and their role in the treatment of a variety of malignancies is currently being explored (for reviews see Borden, 1984; Krown, 1986; Golomb, 1986). This chapter reports on studies examining the effects of IFN on the growth of the human HCC cell line PLC/PRF/5 both in vitro and when established as a tumour xenograft in athymic mice, and also reports on the possible mechanisms whereby interferons may exert cytotoxic effects in this animal model.

MATERIALS AND METHODS

Interferon

All studies were performed using human lymphoblastoid IFN (Hu IFN-α[Ly], "Wellferon", Wellcome Research Laboratories, Beckenham, UK). This product is a mixture of at least eight α-interferons and is produced by the stimulation of suspension cultures of the Namalwa lymphoblastoid cell line by Sendai virus.

In vitro studies

The PLC/PRF/5 cell line was used throughout and cultured as described in Chapter 4. For these experiments, cells were harvested by mild trypsinisation and suspended in modified Eagle's medium (MEM) at a concentration of 1×10^5 cells/ml. Two hundred µl aliquots were dispensed into flat-bottomed microtitre plates (Nunc). Interferon was diluted in MEM and added to the wells to yield final well concentrations of IFN ranging from 1.25 to 5×10^3 IU/ml. Interferon

which had been inactivated by incubation with 0.1 mg/ml of trypsin for one hour at 37°C was used as an internal control. Cells were incubated with IFN for times ranging from 24 to 72 hours and then one μCi of tritiated thymidine ([^{3}H] thy: Amersham International PLC, UK) was added to each well. After a further 18 hour incubation, cells were washed three times to remove non-incorporated radioactivity and harvested by trypsinisation and aspiration onto glass fibre discs by means of a Titertek cell harvester (Flow Laboratories Ltd, UK). Discs were air-dried and radioactivity counted. Each experiment was performed in triplicate.

Animal studies

Tumour xenografts derived from the PLC/PRF/5 cell line were grown in athymic mice as described in Chapter 4. Mice with established growing tumours of base area 20-60 mm^2 were allocated to receive subcutaneously daily either 200 μl of sterile phosphate-buffered saline (PBS), 200 μl of PBS containing 1 x 10^5 IU of IFN or 200 μl of PBS containing 2 x 10^5 IU of IFN. Each group contained eight animals and they were treated daily till death. Tumour size was assessed weekly by measuring tumour base area and by measuring serum AFP concentrations, as described in Chapter 4.

In a separate experiment designed to examine the mechanisms by which IFN might be cytotoxic in this animal model, two groups, each containing five mice with growing tumour xenografts, were treated subcutaneously daily with either 200 μl of PBS containing 2 x 10^5 IU of IFN or 200 μl of PBS alone. At the end of 14 days treatment, all animals were sacrificed and their tumours and livers immediately excised.

A portion of each tumour was fixed in 10% formaldehyde solution and embedded in paraffin for histological examination. The remaining tumour and all specimens of liver were snap-frozen in liquid nitrogen and stored at -70^{o}C until examined.

Cryostat sections, 5 μm thick, were cut from snap-frozen specimens of tumour and liver and examined for HLA class I glycoprotein display using an indirect immunofluorescence technique. After fixation in chloroform/acetone solution (1:1 v/v), sections were washed in PBS for 15 minutes and then incubated for 30 minutes at room temperature in a humidified chamber with 15 μl of either W6/32, an anti-HLA class I glycoprotein monoclonal antibody (Sera-Lab, Sussex, UK) (Barnstaple et al, 1978), or mouse serum. Sections were then washed in PBS for 30 minutes and 15 μl of fluorescein-conjugated rabbit anti-mouse IgG (Dakopatts, Bucks, UK) diluted 1:20 was added as second layer reagent for a further 30 minutes. Following a final 30 minute wash in PBS, slides were mounted in PBS-glycerol with p-phenylene diamine and examined using a Zeiss fluorescence microscope equipped with an IV/2 epifluorescence condenser and Zeiss photometer 03, HLA class I glycoprotein staining intensity being measured semi-quantitatively as previously described by Montano et al (1982). The result for each section was a mean of the measurements of staining intensity from eight randomly selected areas on each slide.

The activity of the enzyme 2,5-oligoadenylic acid (2,5 A) synthetase, a potent inhibitor of protein synthesis, was measured in tumour xenografts and mouse liver using a modification of the method of Minks et al (1979) as follows. Tissue extracts were prepared by

homogenising samples in a buffer containing 0.5% Nonidet-P40, 7 mM 2-mercaptoethanol and 20 mM Hepes, pH 7.5. The homogenate was centrifuged and the supernatant removed. Protein concentration was measured by the method of Lowry et al (1951) and the supernatant was then stored at -70°C until assayed. One hundred µl of thawed extract was added to 50 µl of poly (rI):poly (rC)-agarose beads (PL Biochemicals, UK) which had been equilibriated in buffer A: 10 mM Hepes, pH 7.6, 50 mM KCl, 2 mM $Mg(OAc)_2$, 7 mM 2-mercaptoethanol, and 20% glycerol. Beads were incubated with tissue extracts for 30 minutes at 30°C and then washed three times in buffer A. Beads were then incubated for 20 hours at 30°C in 200 µl of a reaction mixture containing 20 mM Hepes, pH 7.6, 40 mM KCl, 25 mM $Mg(OAc)_2$, 7 mM 2-mercaptoethanol, 5 mM ATP, 10 mM creatine phosphate, 0.32 mg/ml creatine kinase, 0.1 mg/ml poly (rI):poly (rC) (all Sigma Chemical Co Ltd, UK) and 0.1 µCi [^{3}H] ATP (Amersham International PLC, UK). The reaction was terminated by heating to 90°C for five minutes. [^{3}H]-labelled 2,5A was then purified by DEAE cellulose chromatography and the radioactivity of the entire sample was measured after dilution in a liquid scintillant.

Statistical analysis

The unpaired Student's t-test and the log-rank test were used as appropriate. Survival curves were drawn up by the Kaplan-Meier method.

RESULTS

Effect of IFN on [^{3}H] thymidine uptake by PLC/PRF/5 cells

The uptake of [^{3}H] thymidine by PLC/PRF/5 cells was inhibited by IFN in a dose dependent manner (Figure 5 (i)). The degree of inhibition noted after 24 hours incubation with IFN could not be increased by more prolonged incubation (data not shown). Inhibition of DNA synthesis was noted at the lowest IFN concentration studied, 1.25 IU/ml, and a 50% inhibition of [^{3}H] thymidine uptake was noted at IFN concentrations of around 1000 IU/ml. In contrast, trypsin-inactivated IFN had no effect on [^{3}H] thymidine uptake by PLC/PRF/5 cells.

Effects of IFN on HCC xenograft growth

As assessed by measuring tumour base area (Table 5 (i)) or serum AFP concentration (Table 5 (ii)), IFN produced a dose-dependent inhibition in the growth of HCC xenografts in athymic mice. In the animals treated with 2×10^5 IU/day of IFN, tumours grew significantly more slowly than those in control animals. Interferon also increased the length of survival of tumour bearing animals (Figure 5 (ii)). Again, the effect was dose-related, and the duration of survival of mice treated with 2×10^5 IU/day of IFN was significantly longer than that of control animals ($p=0.03$).

Effects of IFN on HLA class I glycoprotein display, tumour histology and 2,5A synthetase activity

HLA class I glycoprotein display was increased approximately six-fold by IFN treatment. Immunofluorescence intensity increased from 4.2 $\pm$ 0.3 arbitrary units in control animals to 24.8 $\pm$ 0.6 units in the tumours of IFN-treated animals (mean $\pm$ SEM, $p<0.05$). Apart from size

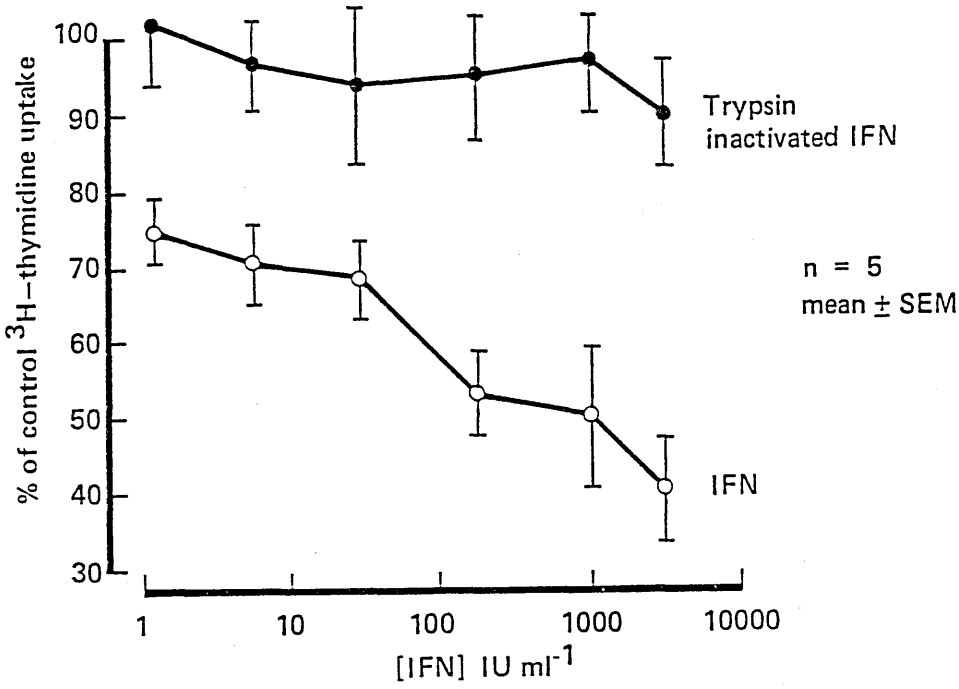

Figure 5 (i)

EFFECT OF IFN ON THE UPTAKE OF [^{3}H] THYMIDINE BY PLC/PRF/5 CELLS. Results are expressed as the mean ± SEM of 5 experiments. Control [^{3}H] thymidine uptake is the uptake seen in cells incubated in the absence of IFN.

Table 5 (i)

TUMOUR GROWTH RATES OF CONTROL AND IFN-TREATED MICE.

Treatment group	Rate of increase in tumour base area (mm^2/week) (mean ± SEM)
Control	29.2 ± 5.28
IFN 1 x 10^5 IU/day	23.7 ± 8.52[a]
IFN 2 x 10^5 IU/day	13.8 ± 4.22[b,c]

[a] p = 0.59 vs control growth rate

[b] p = 0.04 vs control growth rate

[c] p = 0.32 vs IFN 1 x 10^5 IU/day growth rate

Table 5 (ii)

WEEKLY SERUM AFP CONCENTRATIONS (IU/ml) OF CONTROL AND IFN-TREATED MICE DURING THE FIRST FOUR WEEKS OF STUDY (mean ± SEM).

Weeks of therapy	Controls	IFN (1×10^5 IU/day)	IFN (2×10^5 IU/day)
0	2236 ± 1141	1576 ± 557	1928 ± 682
1	4804 ± 1699	2751 ± 973	2337 ± 826
2	8315 ± 2939	3704 ± 1017	2914 ± 883
3	18378 ± 5244	10018 ± 3350	5425 ± 3086[a]
4	40168 ± 12231	36723 ± 11127	10016 ± 5107[a]

[a] $p<0.05$ vs control group

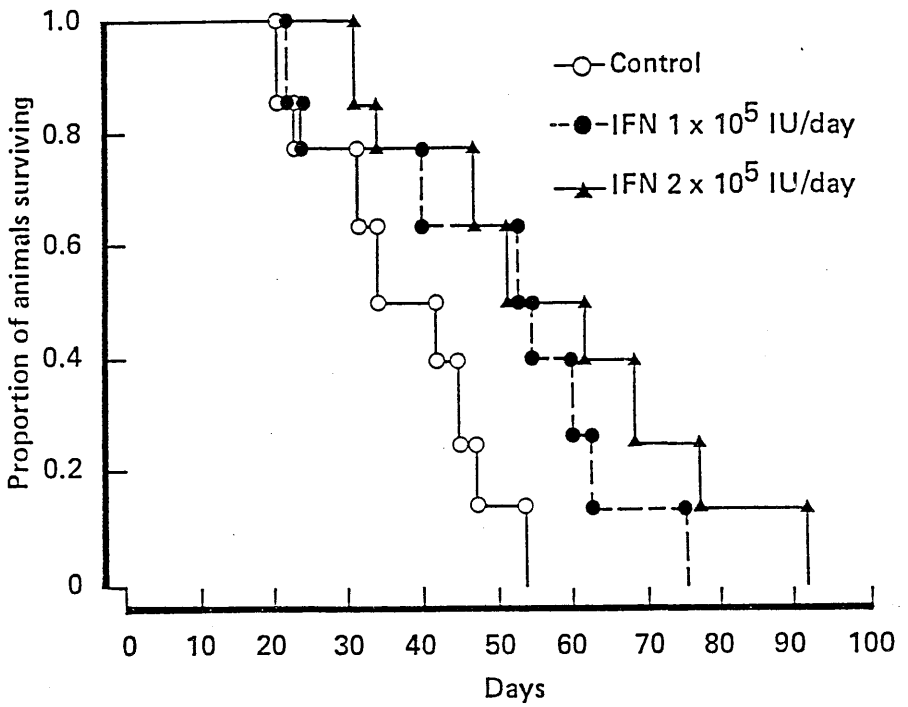

Figure 5 (ii)

KAPLAN-MEIER SURVIVAL CURVES OF CONTROL AND IFN-TREATED MICE.
The median duration of survival of animals treated with IFN 1 x 10^5 IU/day was not significantly longer than the control group (53 vs 43 days, p = 0.08). Those treated with 2 x 10^5 IU/day of IFN survived significantly longer than controls (61 days, p = 0.03).

differences, there were no apparent macroscopic or light-microscopic differences between the tumours of control and IFN treated animals. In particular there were no obvious differences in the degree of local invasivness, the intensity of the inflammatory cell infiltrate or the degree of tumour necrosis. IFN produced a marked rise in 2,5A synthetase activity in the tumours of treated animals (Figure 5 (iii), $p<0.05$). The slight increase in enzyme activity observed in the livers of treated animals did not achieve statistical significance.

DISCUSSION

These experiments demonstrate that human lymphoblastoid IFN produces dose-dependent inhibition of the *in vitro* and *in vivo* growth of the PLC/PRF/5 cell line. The *in vitro* data indicate that α-IFN is capable of a direct inhibitory effect on cell growth, which is manifest even at low IFN concentrations, a finding previously noted by Desmyter et al (1981) and recently confirmed by Motoo et al (1986), who, like Hinoue (1985), also noted *in vitro* sensitivity to β-IFN. *In vivo*, a daily α-IFN dosage of 2×10^5 IU/day was found able to inhibit tumour growth and prolong the survival of mice with PLC/PRF/5-derived tumour xenografts. This dose, as assessed by the calculations of Freireich et al (1966), which take into account species differences in surface area to weight ratio, is equivalent to approximately 20×10^6 IU/day in man, and is far in excess of the thrice weekly dosage of 10^5 IU which Desmyter et al (1981) found incapable of inhibiting the development of HCC xenografts in nude mice injected with PLC/PRF/5 cells. Similarly, the use by Hinoue (1985) of only 2×10^4 IU/day of β-IFN may explain his finding of only brief initial inhibition of PLC/PRF/5 tumour xenograft growth.

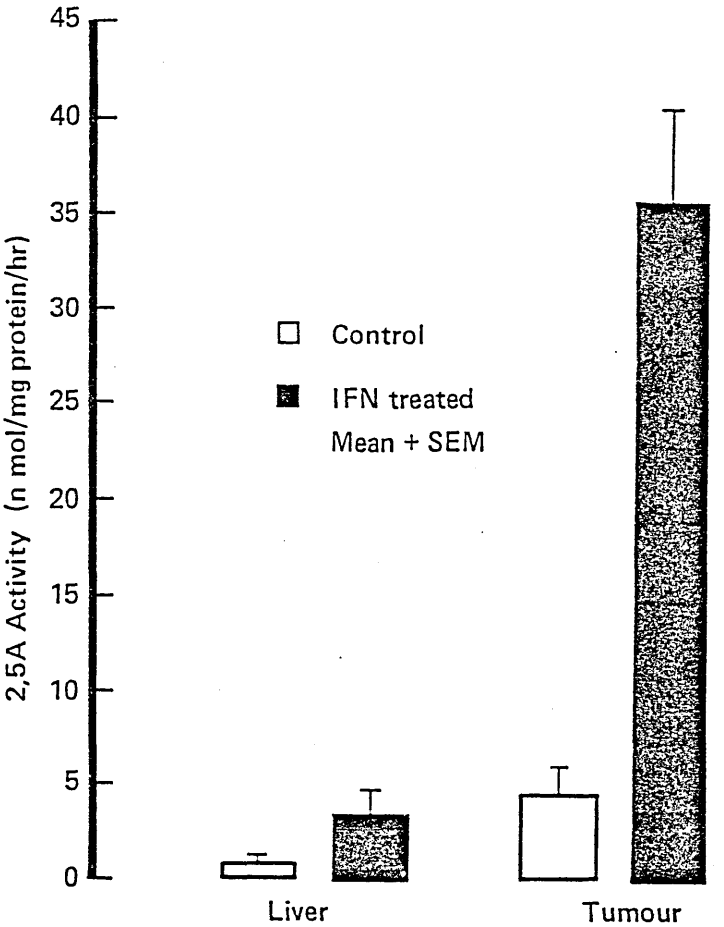

Figure 5 (iii)

LIVER AND TUMOUR 2,5A SYNTHETASE ACTIVITIES IN CONTROL AND IFN-TREATED MICE.

These studies, and those of others (Balkwill et al, 1983), suggest that in the athymic mouse system, the growth of human tumour xenografts is primarily inhibited by the direct antiproliferative effects of human IFN on cell growth. Human α-IFN was found to be capable of producing large increases in the activity of the enzyme 2,5A synthetase only in tumour xenografts and not in mouse tissue, thus demonstrating the potential of this IFN preparation to inhibit tumour growth directly, by inhibition of tumour protein synthesis.

The role of the immunomodulatory effects of IFNs on the growth of human tumours is difficult to study in the athymic mouse because of its congenitally deficient immune system (Kindred, 1978) and because of the HLA incompatibility between mouse and human tissue. Though IFN produced significant increases in the display of HLA class I glycoprotein in tumour xenografts, for the reasons previously stated, it is unlikely that this mechanism played a role in the observed growth inhibitory effects of IFN on tumour xenografts. However, in an immunocompetent and HLA-matched system, it is possible that the enhancement of tumour HLA class I glycoprotein display by IFN, thus making malignant cells more susceptible to immune lysis by T-cell mediated pathways (Zinkernagel and Doherty, 1979), may assume some importance in tumour growth inhibition. It is of note that IFNs, as assessed by serum β_2-microglobulin levels, are capable of increasing HLA class I glycoprotein display in cancer patients (Borden et al, 1982; Lucero et al, 1982).

Though athymic mice have a T-cell deficient immune system, they have a normal population of Natural Killer (NK) cells (Herberman, 1978), and

it has been previously shown that athymic mouse NK cell activity is modulated by mouse IFN, and that this system is important in inhibiting the tumourogenicity of PLC/PRF/5-derived tumour xenografts (Shouval et al, 1983). Though the effects of IFN on mouse NK cell activity have not been examined, it is unlikely that IFN-induced enhancement of NK cell activity was important in producing the inhibitory effect on tumour xenograft growth noted in this study, as it has been previously shown that human IFN has no effect on athymic mouse NK cell activity (Balkwill et al, 1983). It is of interest however, that in the same study, Balkwill et al (1983) demonstrated that mouse IFN could inhibit the growth of human breast cancer xenografts. This was thought to be primarily due to a stimulating effect of mouse IFN on mouse NK cell activity, as tumour xenograft 2,5A synthetase activity was unchanged by mouse IFN. It is therefore possible that in this animal model the administration of both human and mouse IFNs in combination could produce a greater anti-tumour effect than either alone, both by directly inhibiting tumour xenograft protein synthesis and by enhancing mouse NK activity.

Clinical trials using IFNs for the treatment of HCC in man have begun and their results will be of interest, as both the various immunomodulatory effects of IFNs, which cannot be studied adequately in the immunodeficient athymic mouse model, and their direct antiproliferative actions, may both be operational. In Chapter 6 the effects of IFN on NK cell actvity in patients with HCC are examined and the few small clinical trials so far published examining the effects of IFNs as sole therapy for HCC are discussed in detail in Chapter 2.

CHAPTER SIX

NATURAL KILLER CELL ACTIVITY IN HEPATOCELLULAR CARCINOMA: IN VITRO AND IN VIVO RESPONSES TO HUMAN LYMPHOBLASTOID INTERFERON

ABSTRACT

Natural killer (NK) cell cytotoxicity has been measured in patients with HCC and in appropriate control subjects, and the effects of *in vitro* and *in vivo* administration of human lymphoblastoid interferon (Hu IFN-α[Ly], IFN) on NK cell activity determined.

The NK cell activity of HCC patients was significantly lower than that in patients with cirrhosis or healthy control subjects. Reduced NK cell activity in HCC did not correlate significantly with either serum AFP concentration or patient WHO performance grade. NK cell cytotoxicity in all groups could be increased by prior incubation of effector cells with IFN, but this was significant only in HCC patients, where 10 IU/ml of IFN increased NK cell cytotoxicity from 36.9 $\pm$ 10% to 52.8 $\pm$ 8.2% ($p<0.05$, effector:target ratio = 50:1). Further increases in IFN concentration failed to further increase NK cell activity. NK cell cytotoxicity was measured before and 24 hours after 2.5 mU/m^2 was given subcutaneously to four HCC patients. NK cell cytotoxicity rose from 27.5 $\pm$ 8.6% to 60.9 $\pm$ 5.2% (effector:target ratio = 50:1, mean $\pm$ SEM, $p = 0.05$).

INTRODUCTION

NK cells are large granular mononuclear cells which possess the ability to spontaneously lyse a variety of target cells *in vitro*, and they may play a role in the host defence against tumours and viral infections (Herberman, 1983; Bukowski et al, 1983; Gorelik and Herberman, 1986). NK cell cytotoxicity has been reported to be depressed in patients with advanced cancers (Pross and Baines, 1976;

Takasugi et al, 1977; Kadish et al, 1981; Steinhauer et al, 1982), including HCC (Son et al, 1982; Hirai et al, 1986), though the underlying mechanisms are currently unclear.

In this study the NK cell activity of HCC patients has been measured, and because AFP has potent immunological effects *in vivo* (Murgita and Tomasi, 1975a; Murgita and Tomasi, 1975b;Yamada and Hayami, 1983), the relationship between NK cell cytotoxicity and serum AFP concentration has been examined. In addition, as IFNs play an important role in modulating NK cell activity (Herberman et al, 1979; Minato et al, 1980), and because the IFN/NK cell system appears important in inhibiting the tumourogenicity of HCC cell lines in athymic mice (Shouval et al, 1983), the effects of *in vitro* and *in vivo* IFN administration on NK cell activity in HCC patients has been examined.

PATIENTS AND METHODS

Patients

Seventeen untreated patients with histologically proven HCC (15 of whom had an underlying cirrhosis) were studied, and their NK cell activity was compared with cirrhotic patients with no evidence of HCC (n = 13) and with control subjects (either laboratory staff or relatives of HCC patients, n = 12) with normal serum liver function tests. All groups were equally matched with respect to age (HCC patients 61.5 $\pm$ 11.3 years, cirrhotic patients 55.0 $\pm$ 12.4 years, control subjects 57.0 $\pm$ 16.0 years; mean $\pm$ SD). The types of cirrhosis and the clinical condition (World Health Organisation, 1979) of the patients in the HCC and cirrhotic groups are listed in Table 6 (i). All subjects had serum AFP concentrations measured by

Table 6(i)

TYPES OF CIRRHOSIS, PERFORMANCE GRADES, AND SERUM AFP CONCENTRATIONS IN HCC AND CIRRHOTIC PATIENTS.

	HCC Patients (n = 17)	Cirrhotic Patients (n = 13)
Types of cirrhosis		
Cryptogenic	8	5
HBsAg positive	4	3
Alcoholic	1	4
Other	2	1
Non-cirrhotic	2	0
WHO Performance Grade		
0	2	0
1	7	2
2	5	6
3	2	5
4	1	0
Serum AFP (IU/ml)		
<10	4	10
10 - 100	4	3*
101 - 1000	4	0
>1000	5	0

* all less than 20 IU/ml

radioimmunoassay on the day of assay of NK cell cytotoxicity. As assessed by a variety of imaging techniques, all HCC patients had tumour involving both lobes of the liver, and two patients had metastatic HCC.

Assays of NK cell activity

NK cell cytotoxicity was measured in a short incubation ^{51}Chromium release assay as follows:

Effector Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinised whole blood by centrifugation on Ficoll-Hypaque (Lymphocyte separating medium, Flow Laboratories, UK). Mononuclear cells were collected from the interface, washed three times, and suspended in RPMI 1640 medium supplemented with 10% v/v foetal calf serum, L-glutamine and antibiotics (all Gibco Ltd, UK).

Target Cells

The erythroleukemia cell line K562 was used as target cell throughout. Cells were grown in RPMI 1640 medium supplemented as above, and subcultured once weekly. On the day of assay, cells were harvested by gentle agitation, washed in RPMI 1640, suspended at a concentration of 2.5×10^6/ml and then incubated with $Na_2(^{51}Cr)O_4$ (Amersham International PLC, UK) for one hour at a concentration of 100 µCi/ml of cell suspension. Cells were then washed five times in RPMI 1640 and resuspended at a concentration of 2×10^4 cells/ml.

Assay

Five-hundred µl of ^{51}Cr labelled K562 cells were incubated in 10.5 mm x 63.5 mm test tubes with 500 µl of PBMCs suspended at various concentrations, to yield effector:target ratios of 100:1, 50:1 and 10:1. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for four hours. To calculate spontaneous ^{51}Cr release some tubes in each experiment contained only target cells and medium, and to calculate maximal ^{51}Cr release others contained target cells and 500 µl of 1% Triton-X100 detergent. After incubation, cells were centrifuged at 1500 rpm for five mintues and 500 µl aliquots of supernatant were then removed for gamma counting. Each experiment was performed in triplicate and percentage cytotoxicity calculated according to the formula:

$$\% \text{ cytotoxicity} = \frac{\text{mean test counts} - \text{mean spontaneous counts}}{\text{mean maximum counts} - \text{mean spontaneous counts}} \times 100$$

Spontaneous ^{51}Cr release was always less than 20% of maximal ^{51}Cr release and the variability of triplicate determinations was consistently less than 10%.

In vitro and in vivo effects of interferon

As in Chapter 5, human lymphoblastoid interferon (Hu IFN-α[Ly], IFN; "Wellferon", Wellcome Research Laboratories, UK) was used throughout. The *in vitro* effects of IFN were studied in five patients from each group by pre-incubating their PBMCs (4×10^6/ml) for three hours in concentrations of IFN ranging from 0 - 1000 IU/ml. Cells were then washed and the ^{51}Cr release assay performed as above at an

effector:target ratio of 50:1.

The in vivo effects of IFN were studied in four patients taking part in pilot chemotherapy studies examining the toxicity and efficacy of IFN treatment in HCC. Each patient's NK cell cytotoxicity was assayed as above immediately before and 24 hours after a subcutaneous injection of 2.5 megaunits/m^2 of lymphoblastoid IFN.

Statistical analysis

All assays were performed in triplicate and mean values determined. Experimental results are expressed as mean $\pm$ SEM. All statistical tests were non-parametric. The significance of differences between groups was determined using the unpaired or paired Wilcoxon's rank sum tests as appropriate. The significance of correlations was tested using Kendall's rank correlation test.

RESULTS

Figure 6 (i) illustrates the NK cell cytotoxicity of each group at effector:target ratios of 100:1, 50:1 and 10:1. There was no significant difference in NK cell cytotoxicity between healthy controls and patients with cirrhosis at any effector:target ratio. At all effector:target ratios however, NK cell cytotoxicity was significantly reduced ($p<0.05$) in HCC patients when compared to either cirrhotic patients or healthy subjects (e.g. at 50:1, % cytotoxicity HCC patients = 26.3 $\pm$ 3.7%, cirrhotic patients = 45.6 $\pm$ 2.2%, healthy controls = 57.8 $\pm$ 4.6%. mean $\pm$ SEM). NK cell cytotoxicity in HCC patients did not correlate significantly with either serum AFP concentration (Figure 6 (ii); Kendall's coefficient of correlation = -0.103, $p>0.5$) or WHO performance grade (data not shown; Kendall's

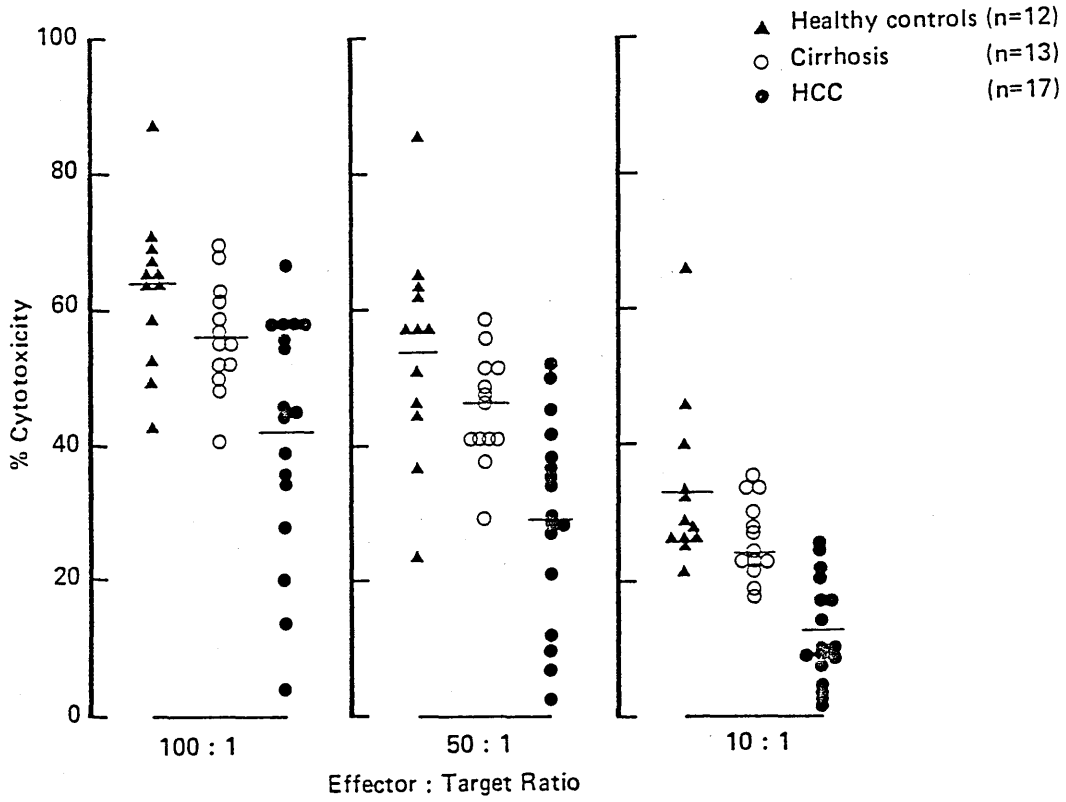

Figure 6 (i)

NK CELL CYTOTOXICITY AGAINST K562 TARGET CELLS OF PERIPHERAL BLOOD MONONUCLEAR CELLS ISOLATED FROM CONTROL SUBJECTS, PATIENTS WITH CIRRHOSIS AND PATIENTS WITH HCC. Bars represent mean values.

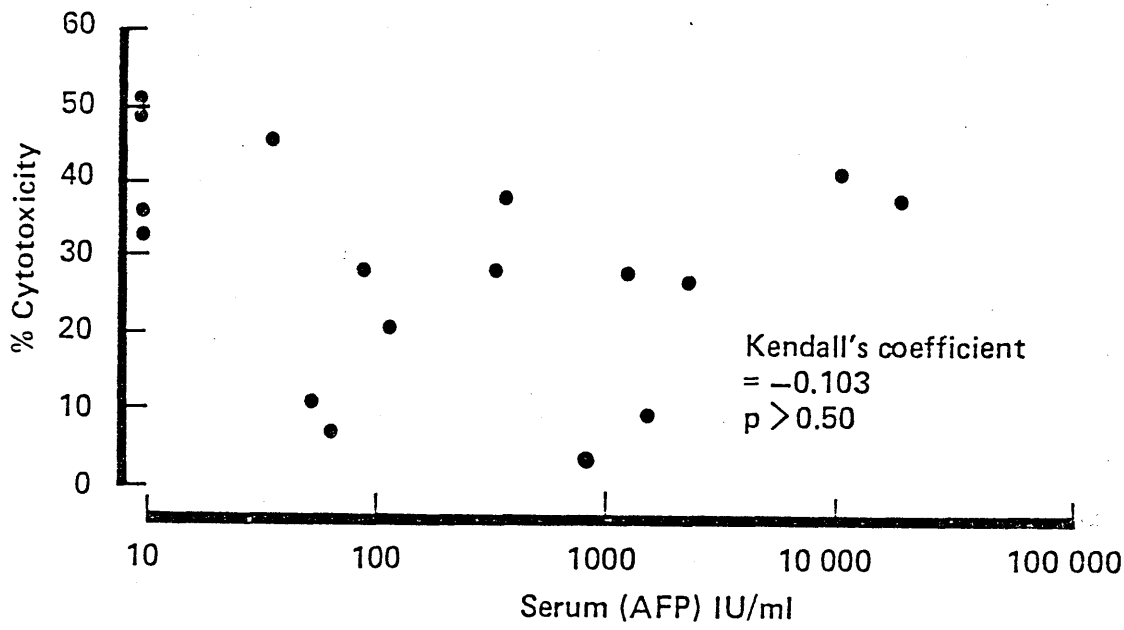

Figure 6 (ii)

SCATTER DIAGRAM OF THE RELATIONSHIP BETWEEN SERUM AFP CONCENTRATION AND NK CELL CYTOTOXICITY (EFFECTOR:TARGET RATIO = 50:1) IN HCC PATIENTS.

coefficient of correlation = +0.15, $p>0.31$). The effect of tumour burden on NK cell cytotoxicity could not be determined as, with the exception of two patients, all had approximately similar tumour burdens.

In vitro, IFN produced increases in NK cell cytotoxicity in all groups (Figure 6 (iii)). At an IFN concentration of 10 IU/ml the percentage increases in NK cell cytotoxicity were greatest in HCC patients ($77.9 \pm 32.4\%$, mean $\pm$ SEM; $p<0.01$ v baseline values). Percentage increases in cirrhotic patients ($13.7 \pm 7.5\%$) and control subjects ($32.8 \pm 16.9\%$) were smaller and the absolute values were not statistically significantly greater than baseline values. Increasing IFN concentration above 10 IU/ml did not result in further large increases in NK cell cytotoxicity in any of the groups.

In the four HCC patients given IFN by subcutaneous injection, NK cell cytotoxicity rose significantly from $27.5 \pm 8.6\%$ to $60.9 \pm 5.2\%$ (effector:target ratio = 50:1, mean $\pm$ SEM, $p = 0.05$; Figure 6 (iv)).

DISCUSSION

The results of this study, using more appropriate control groups, confirm and extend previous observations of diminished NK cell cytotoxicity in HCC patients (Son et al, 1982; Hirai et al, 1986). The aetiology and pathogenesis of this defect are currently unclear. Though NK cell cytotoxicity has been variously reported to be low (Charpentier et al, 1984; James and Jones, 1985), normal (Vierling et al, 1977; Chisari et al, 1981; Serdengecti et al, 1981) or increased (Dienstag et al, 1982) in patients with a variety of chronic liver

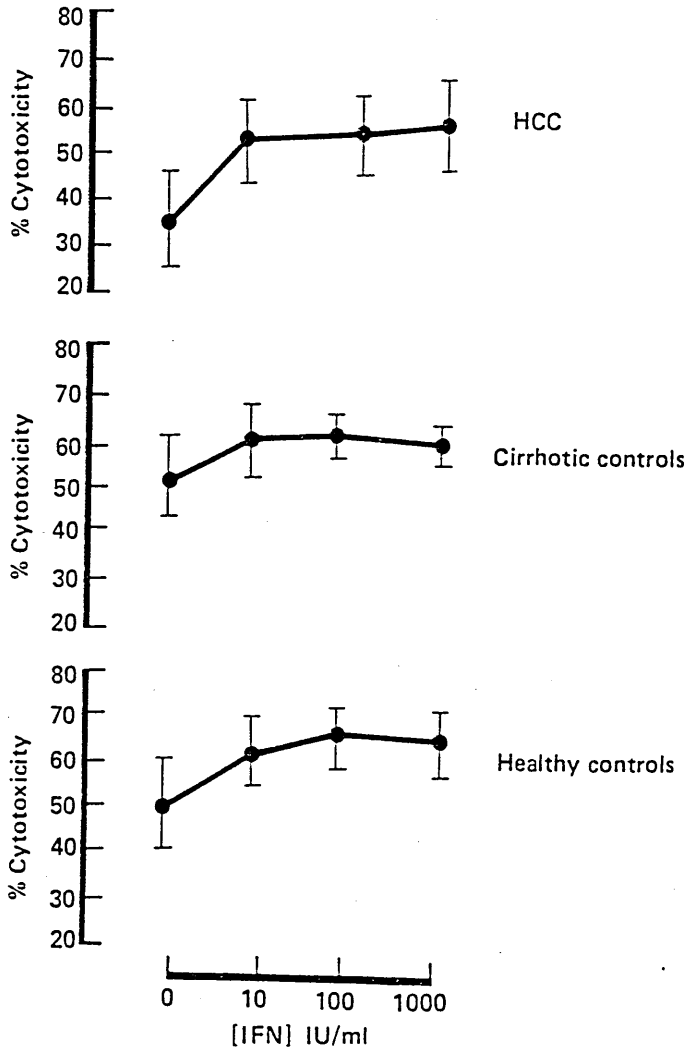

Figure 6 (iii)

THE EFFECT ON NK CELL CYTOTOXICITY OF IN VITRO INCUBATION OF PBMCs WITH HUMAN LYMPHOBLASTOID INTERFERON. Effector:target ratio = 50:1. Mean ± SEM.

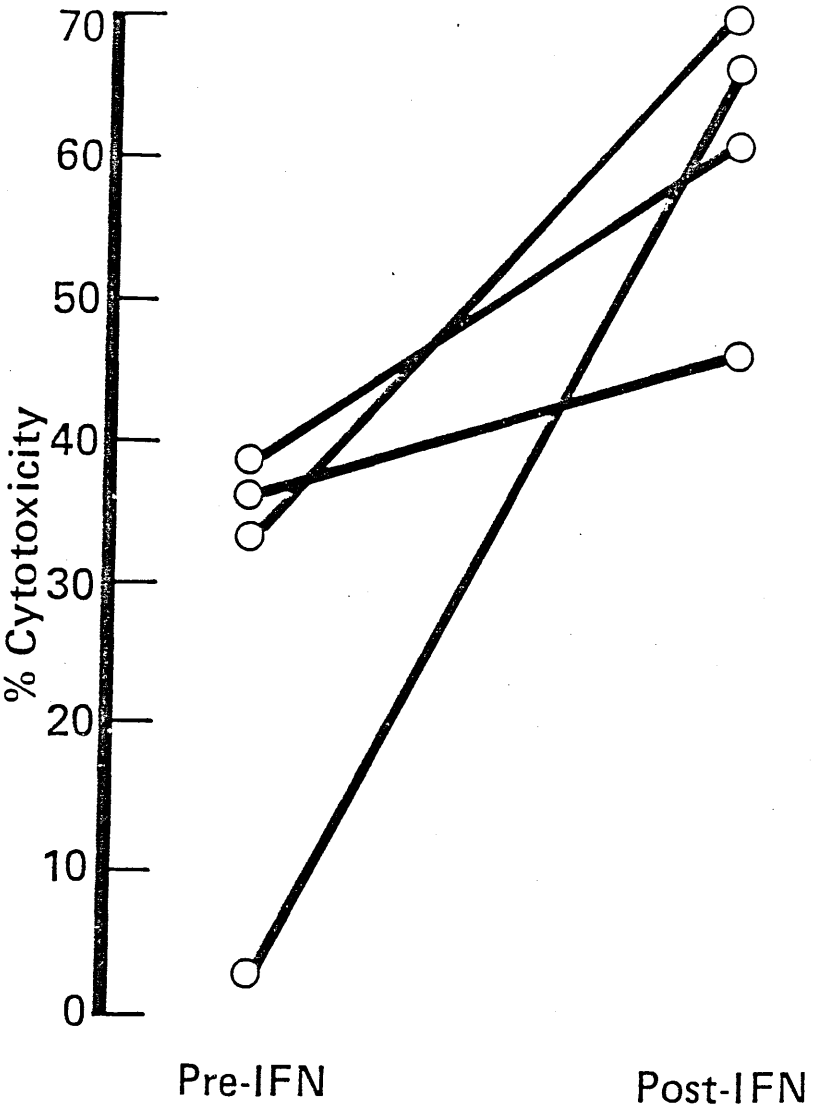

Figure 6 (iv)

THE EFFECT OF IN VIVO ADMINISTRATION OF HUMAN LYMPHOBLASTOID INTERFERON ON THE NK CELL CYTOTOXICITY OF PBMCs ISOLATED FROM HCC PATIENTS (n = 4; effector:target ratio = 50:1).

diseases, it seems unlikely that the low NK cell cytotoxicity in HCC patients is secondary to the presence of underlying chronic liver disease per se, as the control group of patients with broadly comparable chronic liver diseases had normal NK cell activity. Though AFP has potent immunomodulatory effects (Murgita and Tomasi, 1975a; Murgita and Tomasi, 1975b), and may cause reduced NK cell activity in vivo by virtue of the generation of suppressor cells to NK cells (Yamada and Hiyami, 1983), no correlation could be found between serum AFP concentration in HCC patients and their NK cell activity, and it is therefore unlikely that AFP is of major importance in the pathogenesis of the reduced NK cell cytotoxicity in these patients. The possibility that other serum factors may be involved has been examined by Son et al (1982), who found that sera from HCC patients could not inhibit the NK cell cytotoxicity of PBMCs isolated from healthy control subjects.

Reduced numbers of NK cells within the PBMC population of HCC patients cannot be excluded as a cause of the reduced NK cell cytotoxicity observed and further studies are needed which define, using monoclonal antibodies which are relatively specific for cells with NK function (for reveiw see Gorelik and Herberman, 1986), the size of the NK cell population of patients with HCC and chronic non-malignant liver diseases. Normal numbers of circulating NK cells, in the presence of reduced NK cell cytotoxicity, have however been found in studies in patients with advanced cancers (Steinhauer et al, 1982), and in patients with primary biliary cirrhosis (James and Jones, 1985).

Diminished endogenous IFN production (Funa et al, 1983; Funa et al, 1984) and diminished responsiveness of NK cells to IFN (Funa et al,

1984) have been proposed as two of several possible mechanisms resulting in reduced NK cell activity in cancer patients. Though endogenous IFN production has not been measured in this study, it is unlikely that diminished IFN-responsiveness is responsible for the low NK cell activity in HCC patients, as NK cell activity was significantly enhanced both in vitro and in vivo by low doses of IFN. The dose of IFN given to the HCC patients would be expected to produce peak plasma IFN concentrations of only 20-30 IU/ml (Gutterman et al, 1982).

Though it is unclear whether the reduced NK cell cytotoxicity in HCC patients has any deleterious effect on tumour growth and metastatic spread, there is considerable experimental evidence that NK cell activity may be important in host resistance against the growth of primary tumours and metastatic disease (Gorelik and Herberman, 1986). These factors, taken in conjunction with animal studies demonstrating that the IFN/NK cell system is of importance in inhibiting the tumourogenicity and invasiveness of HCC cell lines (Shouval et al, 1983), and the finding that IFN can prolong the survival of mice with xenografts derived from HCC cell lines (see Chapter 5), suggest that IFNs, by virtue of their direct antiproliferative and immunomodulatory effects, may be worthy of evaluation in the treatment of HCC. As discussed in detail in Chapter 2, preliminary results in small numbers of patients have already been reported. While immunostimulation has been noted during IFN treatment for HCC (Nair et al, 1985), responses to therapy have so far been very disappointing (Nair et al, 1985; Sachs et al, 1985; Forbes et al, 1985).

CHAPTER SEVEN

HUMAN HEPATOCELLULAR CARCINOMA TUMOUR XENOGRAFTS: THEIR ANDROGEN RECEPTOR STATUS AND GROWTH RESPONSES TO CASTRATION

ABSTRACT

Castrated or sham-operated male athymic mice were inoculated with cells from the human hepatocellular carcinoma cell line PLC/PRF/5. Fewer castrated than control animals developed tumours and their tumours appeared at later times and grew more slowly, though the small differences noted did not achieve statistical significance. Androgen receptors were assayed in nuclei obtained from three separate liver cancer cell lines and from normal adult human liver. Similar concentrations ranging from 235–550 fMol mg^{-1} DNA of nuclear androgen receptors were detected in all tissues. Low percentages of androgen receptors were retained on DNA-cellulose. Although the presence of receptors implies the potential for metabolic effects of androgens in normal and malignant liver, our in vivo studies suggest that castration does not alter significantly the development and growth of hepatocellular carcinoma xenografts in athymic mice.

INTRODUCTION

For many years there has been strong evidence from animal studies that androgenic steroids may play a role in the development of spontaneous and chemically induced HCCs (Andervont, 1951; Vesselinovitch and Mihailovich, 1967; Reuber, 1969), and there is circumstantial evidence in man that androgenic steroids may play a role in the development of HCC. This is evidenced by the multiple case reports of HCC developing in patients on long-term androgen therapy (Barnstein et al, 1971; Johnson et al, 1972; Bruguera, 1975; Boyd and Mark, 1977), and by the fact that in all areas of the world HCC is a disease with a definite male preponderance (Peters, 1976).

Recently this evidence has been further strengthened by the finding of androgen receptors in human HCC tissue (Iqbal et al, 1983; Nagasue et al, 1985; Ohnishi et al, 1986). It is therefore possible that androgens may exert metabolic effects on human HCC in vivo, and that androgenic manipulation may play some role in the treatment of this malignancy.

In this chapter the effects of castration on the development and growth of human HCC tumour xenografts in athymic mice are reported, as are androgen receptor measurements in normal adult human liver and in tumour xenografts derived from three different human HCC cell lines.

METHODS

Effects of castration

Sexually mature male athymic mice were either castrated or had sham surgery (scrotal incision and suturing). After seven days of antibiotic treatment, serum testosterone levels were measured by radioimmunoassay (Dyas et al, 1979) in the 11 animals in each group. Mice were then irradiated and inoculated with PLC/PRF/5 cells as described in Chapter 4, and observed weekly for evidence of tumour development. Tumour growth was then measured weekly for four weeks by using precision calipers and by measuring serum AFP concentration (Chapter 4).

Androgen receptor analyses

Tumour xenografts of the PLC/PRF/5, SK-Hep-1 and Mahlavu cell lines were grown in male athymic mice as previously described and allowed to grow until they weighed at least 1.5 grams. Animals were then

sacrificed and the tumours immediately excised and frozen at -70°C. Normal adult liver was obtained for analysis from two brain-dead subjects (one male, one female) donating organs for transplantation, and tissue was likewise frozen at -70°C until assayed, usually within two weeks of collection.

Nuclei were purified by the method of Davies and Griffiths (1975). Specific androgen receptor (AR) sites were assayed in whole nuclei using (1, 2, 4, 5-^{3}H) 5 α-dihydrotestosterone ([^{3}H] DHT: 110 Ci/mMol, Amersham International PLC, UK) as ligand by the protamine sulphate precipitation method of Davies et al (1977). Concentrations of AR were calculated as fMol of [^{3}H] DHT specifically retained per mg nuclear DNA. DNA was estimated throughout by the method of Burton (1956). The preparation of DNA-cellulose was carried out as described by Alberts and Herrick (1971) with minor modifications. Salmon testis DNA (1.5 mg/ml) was mixed with extensively washed, neutralised, lyophilised Whatman F11 cellulose (approx. 0.5 g/ml) to generate a thick paste which was lyophilised, ground, dried thoroughly, suspended in tris-HCl (10 mMol/l, pH 7.4) containing EDTA 1 mMol/l, washed twice in this buffer to remove free DNA and then stored as a frozen slurry in this buffer containing NaCl (150 mMol/l) until used. The average incorporation of DNA was 480 µg per packed ml, i.e. 33% of the input DNA was adsorbed onto cellulose.

Analysis of the DNA-binding capacity of AR was carried out at 4°C as follows. A slurry of DNA-cellulose diluted 10-fold in tris-HCl (10 mMol/l, pH 7.4) containing EDTA (1 mMol/l) and NaCl (50 mMol/l) was packed into 1 ml volume syringes (diameter 5 mm) to a bed volume of 0.25-0.30 ml under gravity. Columns were equilibrated in tris-HCl (10

mMol/l, pH 7.4) containing EDTA (0.1 mMol/l) dithiothreitol (0.25 mMol/l) and NaCl (50 mMol/l) (buffer A). Receptor preparations, previously labelled with [^{3}H] DHT in the presence and absence of a 100-fold higher concentration of radioinert DHT, were applied to columns in 200 µl. Columns were washed in approximately 30 bed volumes (7.5-9.0 ml) of buffer A until a constant background of radioactivity (360-400 dpm/ml) was obtained. Specifically retained [^{3}H] DHT-AR complexes were then eluted with six successive 500 µl aliquots of buffer A but containing 500 mMol/l NaCl and 0.2 mg/ml bovine serum albumin. Columns were eluted at three ml/hour and 500 µl aliquots were collected into scintillation vials and radioactivity determined.

Statistical analyses

The chi-squared test with Yates correction for small numbers and the unpaired Wilcoxon's rank sum test were used where appropriate.

RESULTS

Effects of castration

Serum testosterone levels were significantly lower in castrated animals than in controls (60 $\pm$ 11 vs 248 $\pm$ 13 ng/l, mean $\pm$ SD, $p<0.01$). One mouse in the castrated group died within two weeks of cell inoculation and was not included in experimental evaluation. There was no macroscopic evidence of tumour development at death.

Seven of the 10 castrated animals (70%) and eight of the 11 mice in the control group (73%) developed tumours (p = not significant). Similarly there was no significant difference between the two groups

in the time between tumour cell inoculation and macroscopic tumour development. Two weeks after inoculation four of the seven tumours in the castrated animals and six of the eight in the control animals had developed (Table 7 (i)). No significant differences existed between the groups with respect to rate of increase in tumour xenograft base area (control animals 49.1 $\pm$ 9.6 mm^2/week vs castrated animals 44.0 $\pm$ 20.5 mm^2/week, mean $\pm$ SD) and at the end of the four week observation period both groups were similar with respect to tumour base area (control animals 164.8 $\pm$ 92 mm^2 vs castrated animals 145.2 $\pm$ 61.5 mm^2, mean $\pm$ SD, p = NS) and serum AFP concentration (control animals 25744 $\pm$ 16813 IU/ml vs castrated animals 21985 $\pm$ 1328 IU/ml, mean $\pm$ SD, p = NS).

Androgen receptor studies

Nuclear AR were found in normal human liver and the HCC xenografts derived from the PLC/PRF/5, SK-Hep-1 and Mahlavu cell lines (Table 7 (ii) and Figure 7 (i)). The AR concentrations for the tumour xenografts fell between those observed for the samples of normal liver. The Kd values for AR in tumour xenografts fall into the range noted for receptors in classically androgen-dependent tissues, but those for normal human liver are somewhat high. The results of the DNA-cellulose binding assay are shown in Figure 7 (ii). The absence of a radioactivity peak during the high molarity salt elution of [^{3}H] DHT-AR complexes which had been incubated in the presence of unlabelled DHT demonstrates the binding specificity of [^{3}H] DHT to AR. To determine functionality of receptors at a preliminary level the capacity to associate with DNA was investigated. High-salt buffers eluted 12.2% of specific receptors from DNA-cellulose indicating that more than 85% of input AR was not retained by DNA.

Table 7 (i)

TIME TAKEN POST TUMOUR CELL INOCULATION TO MACROSCOPIC TUMOUR DEVELOPMENT IN CONTROL AND CASTRATED ANIMALS.

Week	No. of mice with tumours/total no. developing tumours	
	Control	Castrated
1	0/8	0/8
2	6/8	4/7
3	8/8	6/7
4	8/8	7/7

Table 7 (ii)

ANDROGEN RECEPTOR CONCENTRATIONS AND DISSOCIATION CONSTANTS (Kd) FOR ADULT HUMAN LIVER AND EACH HCC CELL LINE.

Tissue	AR (fMol mg^{-1} DNA)	Kd (nmol l^{-1})
Normal liver (male donor)	550	14.7
Normal liver (female donor)	235	7.3
PLC/PRF/5 xenograft	401	5.4
SK-Hep-1 xenograft	375	9.0
Mahlavu xenograft	303	9.6

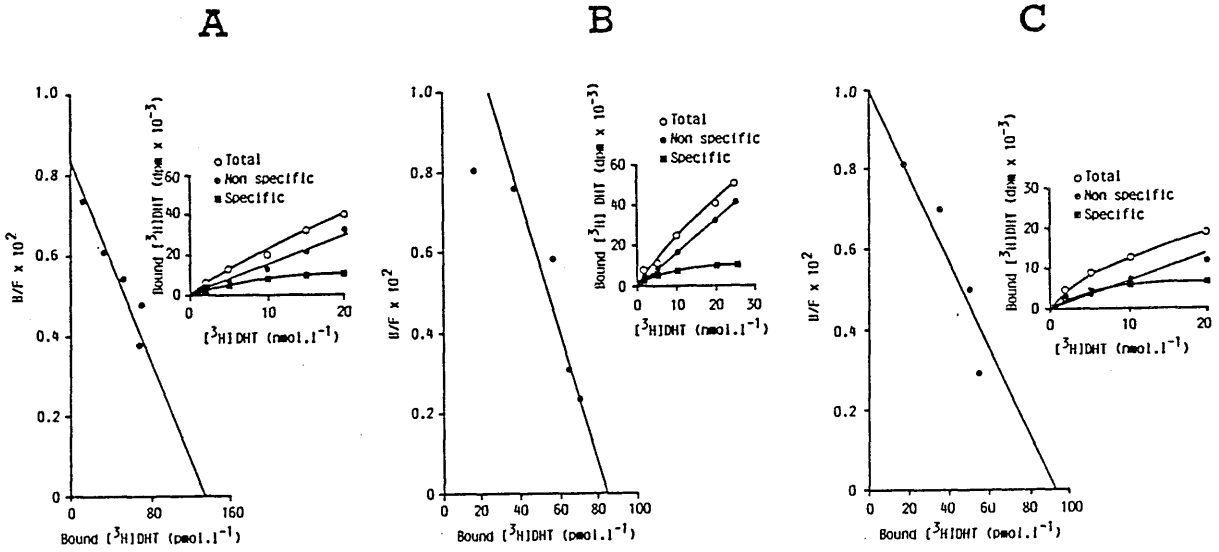

Figure 7 (i)

SATURATION ANALYSIS OF [^{3}H] DHT BINDING TO NUCLEAR AR FROM NORMAL ADULT LIVER (A) AND TUMOUR XENOGRAFTS DERIVED FROM THE PLC/PRF/5 (B), AND SK-HEP-1 (C) CELL LINES.

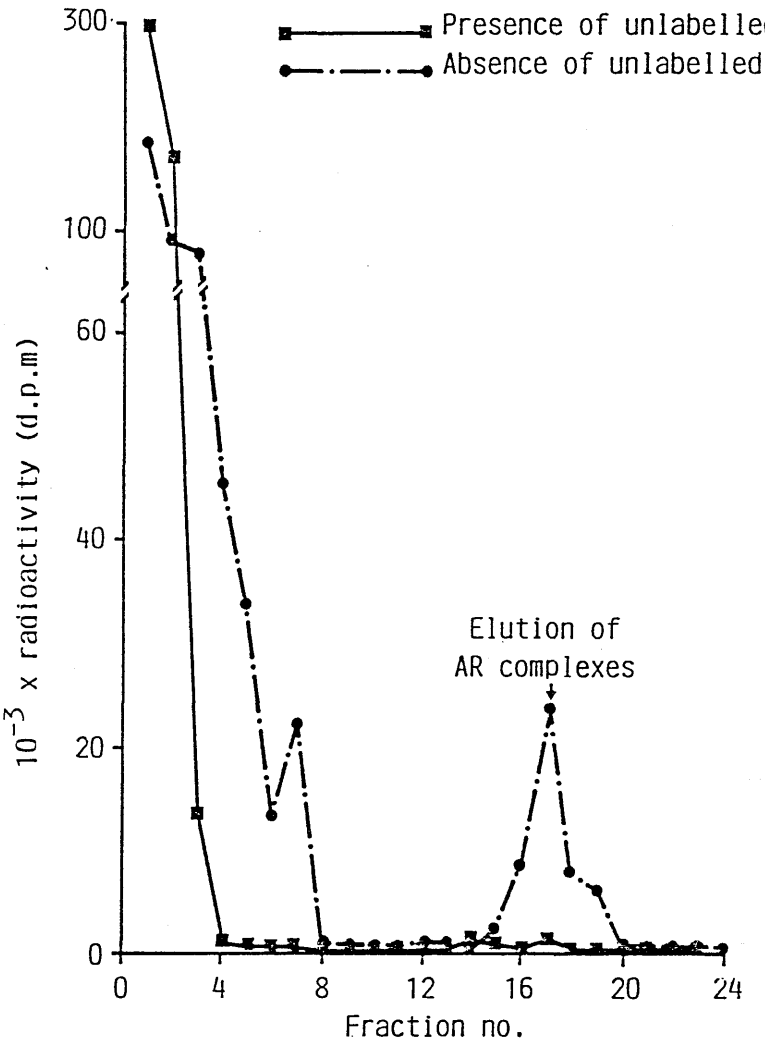

Figure 7 (ii)

ELUTION OF [^{3}H] DHT-AR COMPLEXES (FROM A PLC/PRF/5-DERIVED TUMOUR XENOGRAFT) FROM DNA-CELLULOSE BY 500 mM NaCl.

DISCUSSION

The association of HCC with male sex and with androgenic steroid therapy, and the work by Iqbal et al (1983), demonstrating for the first time the presence of AR in human HCC tissue, all suggest that HCC may be an androgen-responsive malignancy and hence that androgenic ablation many have some role to play in treatment. This study was conducted to establish whether castration could alter tumour development and growth in athymic mice inoculated with cells from a human HCC cell line, and to establish the AR status of human HCC cell lines and normal human liver tissue.

Nuclear AR were detectable both in specimens of normal adult liver and in tumour xenografts from all three human HCC cell lines studied. The finding of nuclear AR in normal adult liver is in contrast to the findings of Iqbal et al (1983), who found no evidence of nuclear or cytosolic AR in specimens from four normal livers, but is not surprising considering that androgenic steroids play an important role in the regulation of hepatic protein synthesis (Chan et al, 1978). The differing results in the two studies may be explained by methodological differences. Dextran-coated charcoal, as used by Iqbal et al (1983), strips steroids from receptors, leading to an underestimation of AR concentration (Ekman et al, 1982). This problem is avoided by use of protamine sulphate precipitation (Davies et al, 1977), which also has the advantages of avoiding the problems of metabolism and the precipitation of sex hormone binding globulin (Steggles and King, 1970). Nagasue et al (1985) have recently reported the presence of AR in normal human liver also, and both he and Ohnishi et al (1986) have been able to detect AR in human HCC

tissue and, less frequently and at lower concentration, in some specimens of surrounding non-neoplastic hepatic tissue.

The nuclear AR concentrations demonstrated in this study are similar in both benign and malignant liver tissue and range from 235-550 fmol mg^{-1} DNA. These values are considerably lower than the nuclear AR levels seen in rat ventral prostate but are similar to those seen in the normal, benign hypertrophic, and malignant human prostate (Davies, 1984). The presence of AR in liver tumour nuclei suggest a receptor-mediated system of androgen action. However, whether the low concentrations of AR observed are sufficient to promote molecular effects remains to be established, and whether the presence of AR represents a totally functional population and at what level requires more investigation. As current dogma implies a primary role of AR at the level of gene expression, the capacity of AR to bind DNA represents a preliminary determination of functional involvement. However, not all receptors in target cell nuclei associate with DNA in a form dissociated by high molarity salt solutions (Barrack et al, 1977; Davies, 1983; Donnelly et al, 1984). In fact, the receptors which associate with nuclear components other than DNA may be more functional as regards stimulation of growth processes (Barrack et al, 1983). The detection of hepatic nuclear AR should not therefore be dismissed because of its low level of binding to DNA, and further work is needed to determine the exact role and biological significance of this finding.

Castration had no significant effect on the development and growth of HCC xenografts in athymic mice. However, there was an apparent trend which suggested that castration may result in fewer tumour

developments and slower growth rates, and it is possible that a significant treatment difference may have been observed had a much larger number of animals been studied.

It is concluded that androgen receptors are present in low concentration in normal adult liver and in HCC cell lines. Though the exact biological significance of this finding is at present unclear, the presence of nuclear AR in established HCC cell lines, and the ease with which these lines can be grown as xenografts in athymic mice provide an excellent model for the further exploration of the effects of androgens and anti-androgenic drugs on HCC development and growth.

CHAPTER EIGHT

IN VITRO AND IN VIVO TUMOUR LOCALISATION WITH A MONOCLONAL ANTIBODY DIRECTED AGAINST A MEMBRANE ANTIGEN ON THE HUMAN HEPATOCELLULAR CARCINOMA CELL LINE PLC/PRF/5

ABSTRACT

A monoclonal antibody, designated K-PLC$_1$, has been produced to a tumour-associated antigen on the cell membrane of the PLC/PRF/5 cell line. Using indirect immunofluorescence techniques, this antibody produced membrane staining of three HCC cell lines and positively stained 10 of 11 human HCC biopsy specimens.

In vitro, ^{125}I-labelled K-PLC$_1$ bound specifically to PLC/PRF/5 cells, as shown by competitive inhibition experiments. Antibody binding could be increased by prior incubation of PLC/PRF/5 cells with human lymphoblastoid interferon, though a significant increase was observed only at high IFN concentrations (1000 IU/ml).

Tumours derived from the PLC/PRF/5 cell line were grown in athymic mice and groups of tumour-bearing animals were injected with either ^{125}I-K-PLC$_1$ or ^{125}I-mouse IgG and then sacrificed at one, four or seven days post injection. Bound radioactivity was counted in a variety of solid organs and in blood. Tumour:liver ratios for K-PLC$_1$ were greater than those for mouse IgG at each time point, the differences being greatest on day four (ratio K-PLC$_1$ 4.4 $\pm$ 0.93, ratio mouse IgG 1.53 $\pm$ 0.60, mean $\pm$ SD, $p<0.05$). The amount of ^{125}I-K-PLC$_1$ bound was greater in the tumour than in any other solid organ, the differences again being maximal on day four. Blood pool radioactvity however, remained high throughout the study period.

Anti-HCC monoclonal antibodies such as K-PLC$_1$ may be of value as immunodiagnostic and immunotherapeutic agents in HCC. The effects of IFNs on HCC tumour-associated antigen display merit further study.

INTRODUCTION

The development of monoclonal antibody technology (Kohler and Milstein, 1975) has led to the detection and characterisation of so-called tumour-associated antigens on malignant cells (Lloyd, 1984). Such antibodies have now been produced to antigens associated with many different types of cancer and much current research in clinical oncology is devoted to the evaluation of these antibodies as possible immunodiagnostic and immunotherapeutic agents (Sikora et al, 1984; Dillman and Royston, 1984; Baldwin and Byers, 1985; Morgan and Foon, 1986; Baldwin and Byers, 1986).

Monoclonal antibodies have recently been produced to HCC-associated cell membrane antigens (Carlson et al, 1985; Shouval et al, 1985; Hu et al, 1986). This chapter reports on the ability of one such antibody, K-PLC$_1$, to react with human HCC cell lines and biopsy specimens, and describes *in vivo* tumour localisation studies performed in athymic mice with HCC tumour xenografts. In addition, experiments have been performed *in vitro* to determine whether human lymphoblastoid interferon can increase display of the antigen recognised by K-PLC$_1$.

MATERIALS AND METHODS

Production and purification of K-PLC$_1$

The production of K-PLC$_1$ and the characterisation of the K-PLC$_1$ antigen are fully described by Wiedmann et al (in press). To produce anti-HCC monoclonal antibodies, BALB/c mice were immunised by three intraperitoneal injections of 5 x 10^6 PLC/PRF/5 cells, repeated at three week intervals. Fourteen days after the last injection mice

received an intravenous booster of 10^6 cells, and their spleens were removed four days later. Splenocytes were fused with the NS-1 mouse myeloma cell line using a modification of the polyethylene glycol (PEG) method of Kohler and Milstein (1975) in which splenocytes and myeloma cells at a ratio of 10:1 were incubated in 40% (w/v) PEG for seven minutes at 37°C. The suspension was then diluted in RPMI 1640 medium (Gibco Ltd, UK) and plated out in HAT medium (Flow Laboratories, UK). When hybrid growth was apparent, supernatants were assayed by indirect immunofluorescence on PLC/PRF/5 cell suspensions (see below) and antibody-secreting clones were isolated by repeated limiting dilution on feeder cells. Of the several antibodies obtained, one, designated K-PLC_1, was selected for further study. This antibody is of IgG_1 sub-type and reacts strongly not only with the PLC/PRF/5 cell line, but with several other carcinoma cell lines also, including those of cervix, bladder and colon (Table 8 (i)). The antigen recognised by K-PLC_1 is of predominantly lipid composition, has a molecular weight of 30,000 daltons, and is immunologically distinct from HBsAg and AFP. K-PLC_1 was grown in bulk either as ascites in pristane-primed BALB/c mice or as supernatants in tissue culture, and pure antibody was obtained by the passage of ascites or supernatant through protein A-sepharose columns (Pharmacia) as described by Ey et al (1978). The pH of the eluate was adjusted to 8.0, antibody concentration estimated by the method of Lowry et al (1951), and adjusted to 1 mg/ml. Antibody aliquots were then stored at -70°C until used.

Table 8 (i)

REACTIVITY OF K-PLC$_1$ WITH NEOPLASTIC CELL LINES OF NON-HEPATIC ORIGIN.

Indirect immunofluorescent staining is graded: - (negative), + (positive) and ++ (strong positive). Data from Wiedmann et al, (in press).

CELL LINE	TISSUE OF ORIGIN	REACTIVITY WITH K-PLC$_1$
HL60	Myeloid	-
KM3	Myeloid	-
Nalm6	B cell	-
HT29	Colon	+
HeLa	Cervix	+
RT4	Bladder (well differentiated)	-
RT112	Bladder (moderately differentiated)	+
FJ	Bladder (anaplastic)	++

HCC cell lines

The PLC/PRF/5, SK-Hep-1 and Mahlavu cell lines were studied. Their source and culture conditions are described in detail in Chapter 4. In vitro experiments were performed on cells from sub-confluent flasks 48 hours following a change of culture medium.

Tissue specimens

Samples of tumour were obtained as needle (n = 9) or operative wedge biopsy specimens (n = 2) from patients with histologically proven HCC. Specimens were coated in OCT compound (Ames Co., UK), snap frozen in pre-chilled isopentane, and stored at $-70^{o}C$ until cryostat sections 5 μm thick were cut for immunofluorescence studies.

Indirect immunofluorescence studies

Indirect immunofluorescence microscopy was performed on suspensions of each HCC cell line as follows. Cells were harvested by incubation and gentle shaking in EDTA and then pelleted by centrifugation. After washing three times in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.05% sodium azide (PBS-BSA), cells were suspended in PBS-BSA at a concentration of 10^{6}/ml and 50 μl incubated with 5 μl of K-PLC_1 for five minutes. After three further washes, cells were resuspended in 50 μl of PBS-BSA and incubated with 10 μl of fluorescein (FITC)-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Bucks, UK) for five minutes. After a final three washes, cells were resuspended in 50 μl of PBS-BSA and mounted on glass slides for examination using a Leitz microscope equipped with epifluorescent illumination.

After washing 5 μm thick, air-dried, and unfixed cryostat sections of liver tissue for 10 minutes in PBS, sections were incubated with K-PLC_1 diluted 1:20 with PBS for 30 minutes. Sections were then washed for 30 minutes in PBS and incubated for the same length of time with a 1:20 dilution of FITC-conjugated rabbit anti-mouse immunoglobulin. After a further 30 minute wash in PBS, sections were mounted in PBS containing 50% v/v glycerol and 0.3M diazobicyclo-octane, and examined with the Leitz microscope. Mouse IgG (Sigma Chemical Co Ltd, Dorset, UK) was also used as a first layer reagent in all immunofluorescence studies in order to provide a specificity control.

In vitro binding studies

To determine whether ^{125}I-K-PLC_1 could bind specifically to PLC/PRF/5 cells, competitive inhibition experiments were performed. K-PLC_1 was iodinated by the Iodogen method to a specific activity of 1.0-1.3 MBq/μg. PLC/PRF/5 cells were harvested, washed, and suspended at a concentration of 10^6/ml in PBS-BSA. Fifty μl aliquots of cell suspension were added to 10.5 mm x 63.5 mm test tubes and then a mixture of 50 μl of a 1:20 dilution of ^{125}I-K-PLC_1 and 50 μl of varying concentrations (from 0-20 μg) of either unlabelled K-PLC_1 or mouse IgG was added. Tubes were vortex mixed and allowed to incubate at room temperature for two hours. Following a further vortex mix, 75 μl aliquots of cell suspension were removed for centrifugation through silicone oil. Cell pellets were then assayed for bound ^{125}I-K-PLC_1 in a γ-counter. Two hour incubation periods were chosen as previous time course experiments had shown maximum antibody binding to HCC cell lines at this time (Figure 8 (i)). ^{125}I-K-PLC_1 binding to Mahlavu and SK-Hep-1 cells was also examined as above.

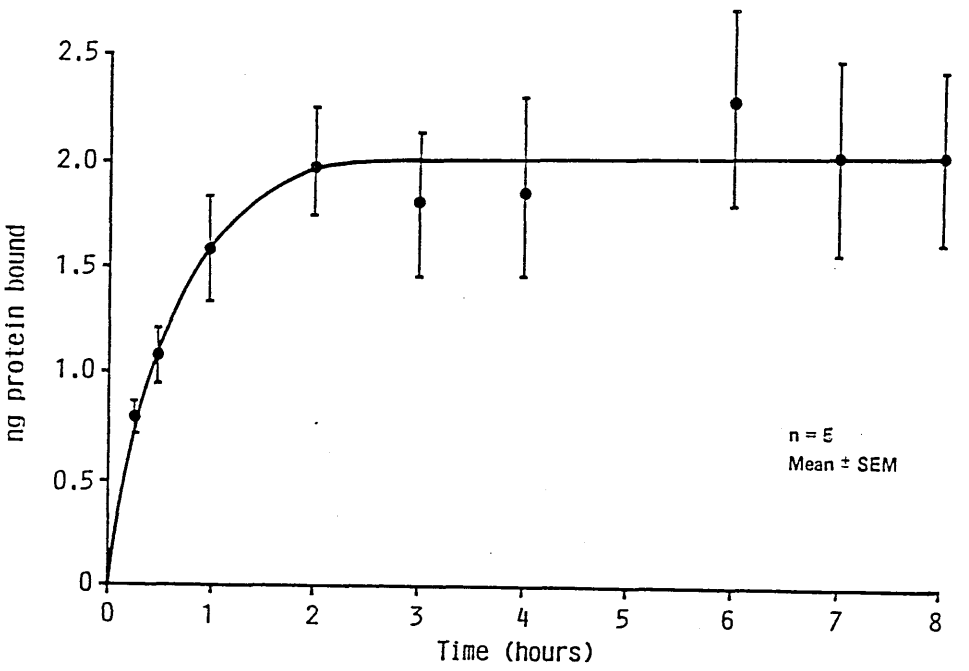

Figure 8 (i)

THE KINETICS OF ^{125}I-K-PLC$_1$ BINDING TO PLC/PRF/5 CELLS. A 1:20 dilution of ^{125}I-K-PLC$_1$ (approx. 62 ng) was incubated with 5 x 10^4 PLC/PRF/5 cells for the times shown. Cell separation was performed by centrifugation through silicone oil. Data represent the mean $\pm$ SEM of five experiments, each done in triplicate.

^{125}I-K-PLC$_1$ binding to PLC/PRF/5 cells was also examined after cells had been cultured in medium containing 0, 10, 100 or 1000 IU/ml of human lymphoblastoid interferon (Hu IFN-α[Ly], IFN, Wellcome Research Laboratories, UK). After a 24 hour incubation in IFN, cells were washed and harvested as above, suspended at 10^6/ml in PBS-BSA, and 50 µl aliquots of cell suspension were incubated with 50 µl of a 1:20 dilution of ^{125}I-K-PLC. After a two hour incubation, cells were separated as above and bound radioactivity counted.

Tumour localisation studies

PLC/PRF/5-derived tumour xenografts were grown in athymic mice as described in Chapter 4, and all localisation experiments were performed in mice with tumours of base area 50-100 mm^2. K-PLC$_1$ and mouse IgG were labelled with ^{125}I by the Iodogen method to specific activities of approximately 1.0-1.3 and 1.2-1.4 MBq/µg respectively and used on the day of preparation. Tumour-bearing mice were injected intraperitoneally with 10 µg of either ^{125}I-K-PLC$_1$ or ^{125}I-mouse IgG. At one, four, and seven days post injection five mice in each antibody group were sacrificed by exsanguination and specimens of tumour, liver, spleen, kidney, stomach, small intestine, heart, lung and skeletal muscle excised. After homogenisation and extensive washing in PBS-BSA, specimens were weighed and bound radioactivity counted. Biodistribution of isotope was expressed as a ratio of counts/min/g in the tumour over counts/min/g in the organ in question.

Examination for circulating K-PLC_1 antigen in the sera of tumour-bearing athymic mice

Five athymic mice were allowed to develop large PLC/PRF/5-derived tumour xenografts. When tumours had reached a base area of greater than 350 mm^2, mice were killed by exsanguination and the sera removed and stored at $-70^{o}C$ until assayed. Sera from five athymic mice without tumours were used as a control. Competitive inhibition experiments were performed as described under in vitro binding studies, using 50 µl of increasing dilutions of mouse serum in place of unlabelled antibody, in order to determine whether mouse serum could inhibit the binding of ^{125}I-K-PLC_1 to PLC/PRF/5 cells. Each experiment was performed in triplicate.

RESULTS

Indirect immunofluorescence studies

K-PLC_1 produced strong membrane staining of each of the three HCC cell lines examined (Figure 8 (ii)). In each case antigen display was heterogeneous in that not every cell stained positively. The percentage of cells in each cell line staining positively with K-PLC_1 was approximately 90% for PLC/PRF/5 cells, and 80% and 65% respectively for the SK-Hep-1 and Mahlavu cell lines.

The results of the immunofluorescence studies on the 11 human HCC biopsy specimens examined are summarised in Table 8 (ii) and a representative example illustrated in Figure 8 (iii). Staining of HCC tissue was membranous and cytoplasmic in site and heterogeneous within

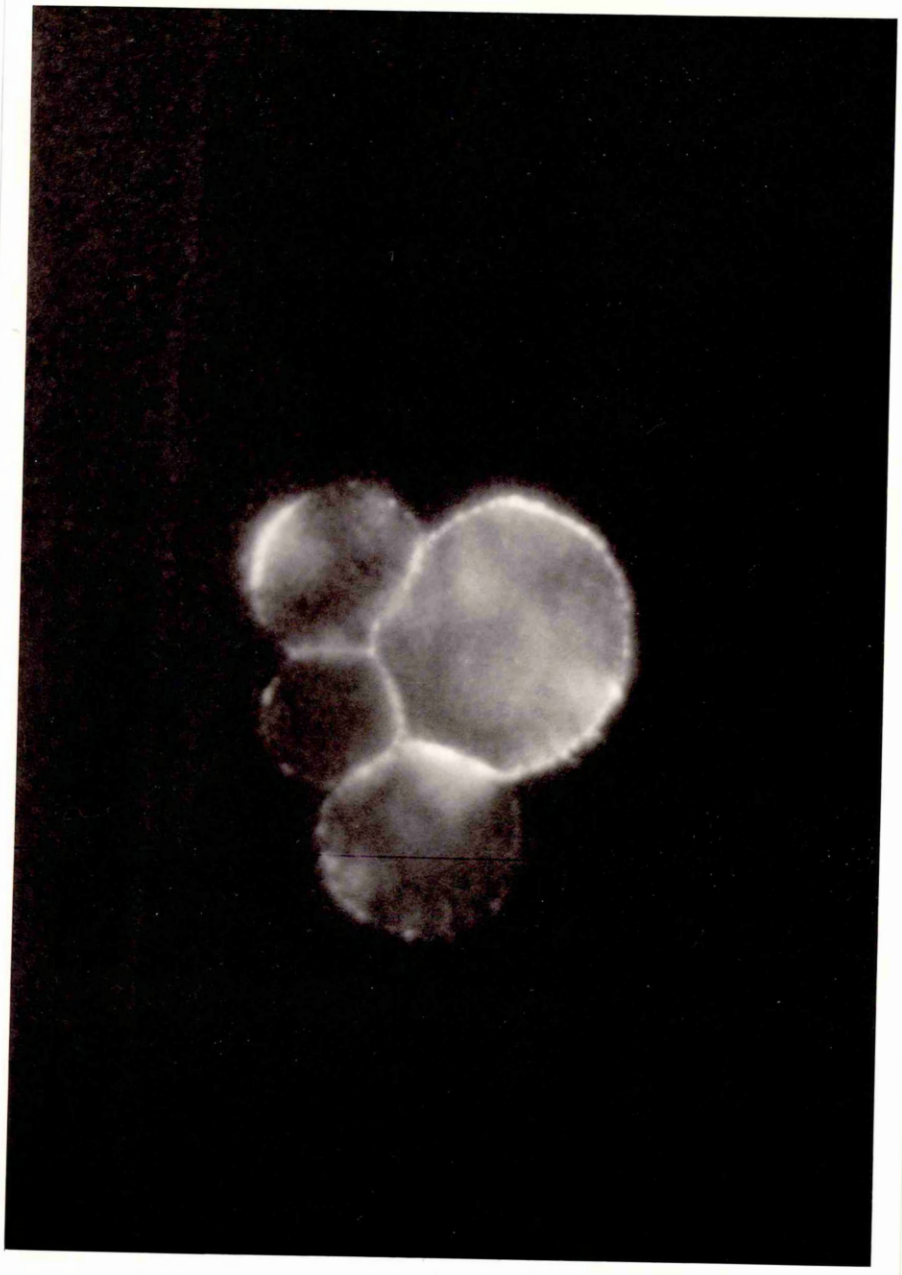

Figure 8 (ii)

INDIRECT IMMUNOFLUORESCENT STAINING OF THE CELL MEMBRANE OF PLC/PRF/5 CELLS BY K-PLC_1 (x200).

Table 8 (ii)

INDIRECT IMMUNOFLUORESCENT STAINING OF HUMAN HCC BIOPSY SPECIMENS: PATIENT CHARACTERISTICS AND STAINING RESULTS.

Staining intensity is graded as in Table 8 (i).

PATIENT	SERUM HBsAg	SERUM AFP (IU/ml)	CIRRHOSIS (TYPE)	REACTIVITY WITH K-PLC$_1$
1	+	14800	+ (Hepatitis-B related)	++
2	+	97300	+ (Hepatitis-B related)	++
3	+	19800	+ (Hepatitis-B related)	++
4	+	67680	+ (Hepatitis-B related)	++
5	-	<10	+ (Primary biliary cirrhosis)	+
6	-	157100	+ (Cryptogenic)	++
7	-	1800	+ (Cryptogenic)	++
8	-	16020	+ (Haemochromatosis)	++
9	-	1740	-	++
10	-	14620	+ (Cryptogenic)	++
11	-	39	+ (Cryptogenic)	-

All patients had tumours of trabecular histological pattern except patients 5 and 8 who had mixed tumours with trabecular and acinar elements.

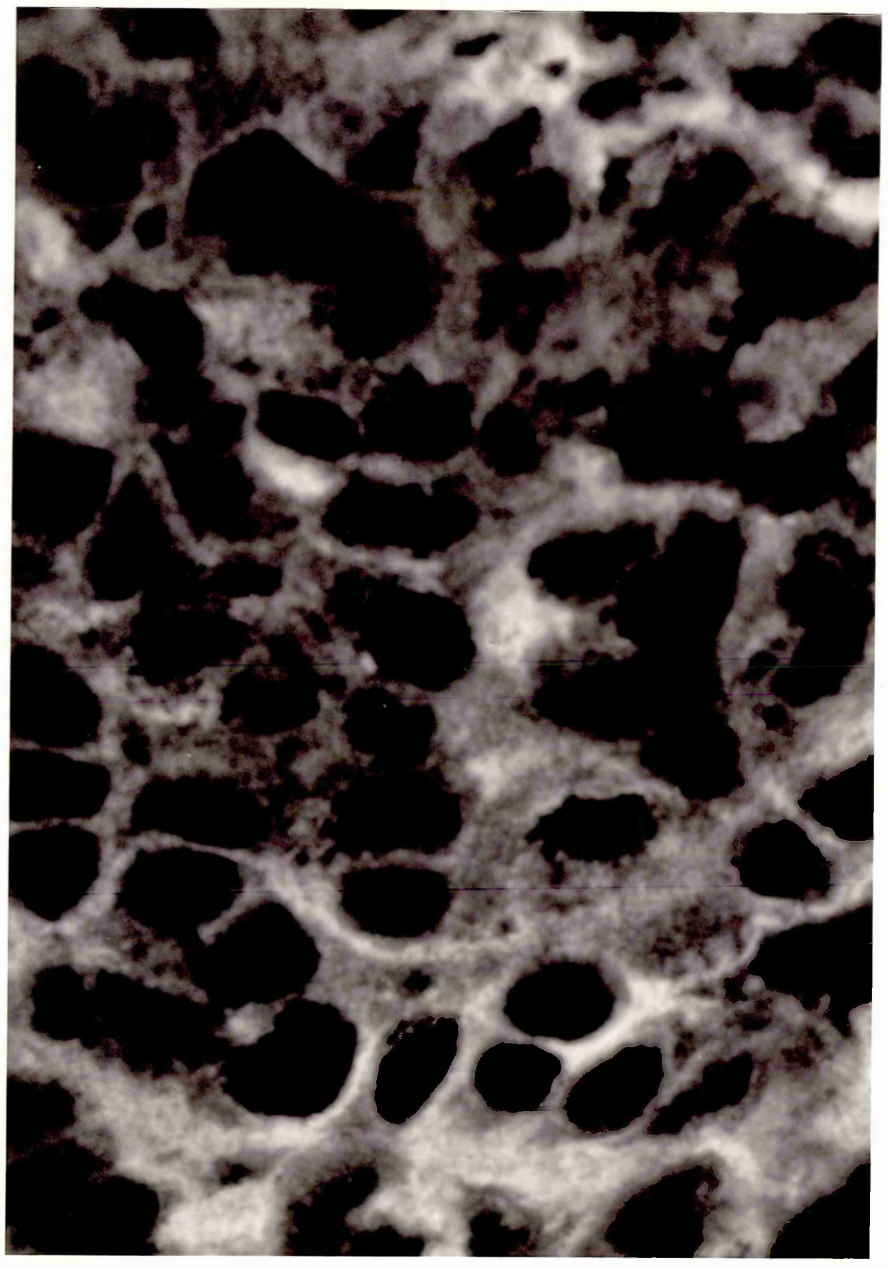

Figure 8 (iii)

INDIRECT IMMUNOFLUORESCENT STAINING OF A HUMAN HCC TISSUE SECTION BY $K\text{-}PLC_1$ (x100).

tumours. One of the 11 tumours did not react with K-PLC_1. This patient had a well-differentiated trabecular pattern HCC arising in a liver with cryptogenic cirrhosis.

In vitro binding studies

Unlabelled K-PLC_1 inhibited the binding of ^{125}I-K-PLC_1 to PLC/PRF/5 cells whereas the control mouse IgG did not (Figure 8 (iv)). ^{125}I-K-PLC_1 bound to each HCC cell line studied (Figure 8 (v)). Approximately 1 ng of antibody bound to 5 x 10^4 PLC/PRF/5 cells and binding to SK-Hep-1 and Mahlavu cells was around 34% and 19% respectively, of that seen with PLC/PRF/5 cells.

Incubation of PLC/PRF/5 cells with IFN for 24 hours prior to the addition of ^{125}I-K-PLC_1 increased antibody binding, suggesting that IFN is capable of increasing the display of the K-PLC_1 tumour-associated antigen (Figure 8 (vi)). This effect was dose-related, but reached statistical significance only at an IFN concentration of 1000 IU/ml ($p = 0.032$, Binomial test). More prolonged incubations with IFN did not increase ^{125}I-K-PLC_1 binding further (data not shown).

Location of HCC xenografts by ^{125}I-K-PLC_1 and examination of mouse sera for K-PLC_1 antigen

K-PLC_1 binds selectively to HCC tumour xenografts growing in athymic mice. At all time points and for all solid organs studied, the tumour:tissue ratios of ^{125}I-K-PLC_1 were greater than 1.0, and far in excess of those seen with the control antibody, ^{125}I-mouse IgG (Figure 8 (vii)). Tumour:tissue ratios of ^{125}I-K-PLC_1 were greatest on day four, with a tendency to decline slightly by day seven post injection.

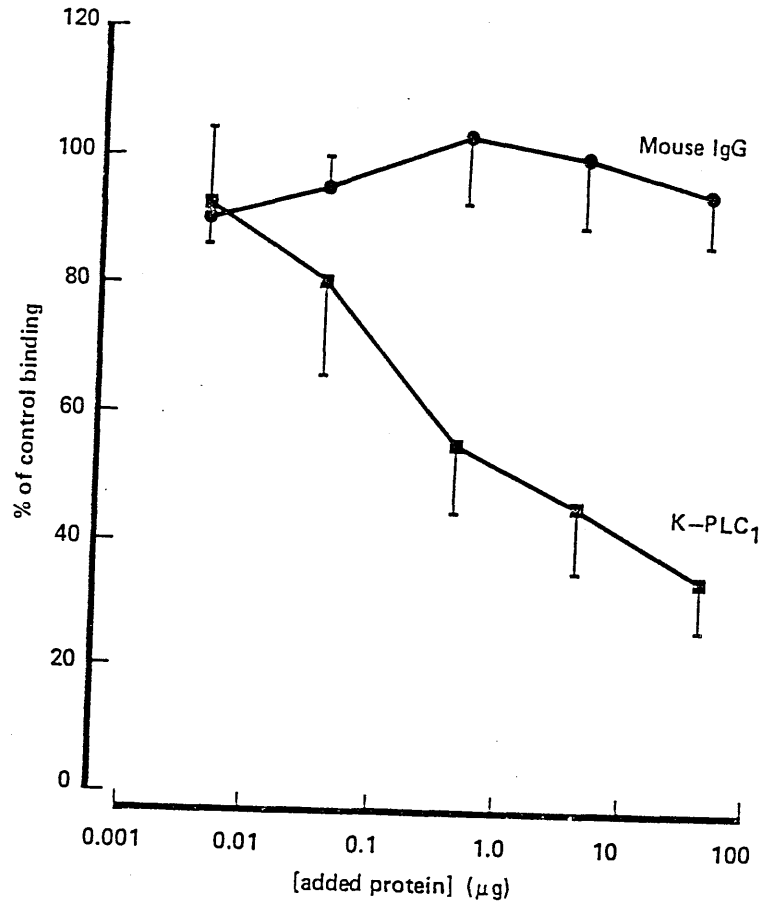

Figure 8 (iv)

COMPETITIVE INHIBITION OF BINDING OF ^{125}I-K-PLC$_1$ TO PLC/PRF/5 CELLS BY INCREASING QUANTITIES OF RADIO-INERT K-PLC$_1$ BUT NOT BY MOUSE IgG. Results are expressed as a percentage of the binding obtained in the absence of any radio-inert antibody and data represent the mean and standard deviation of 5 experiments each performed in triplicate.

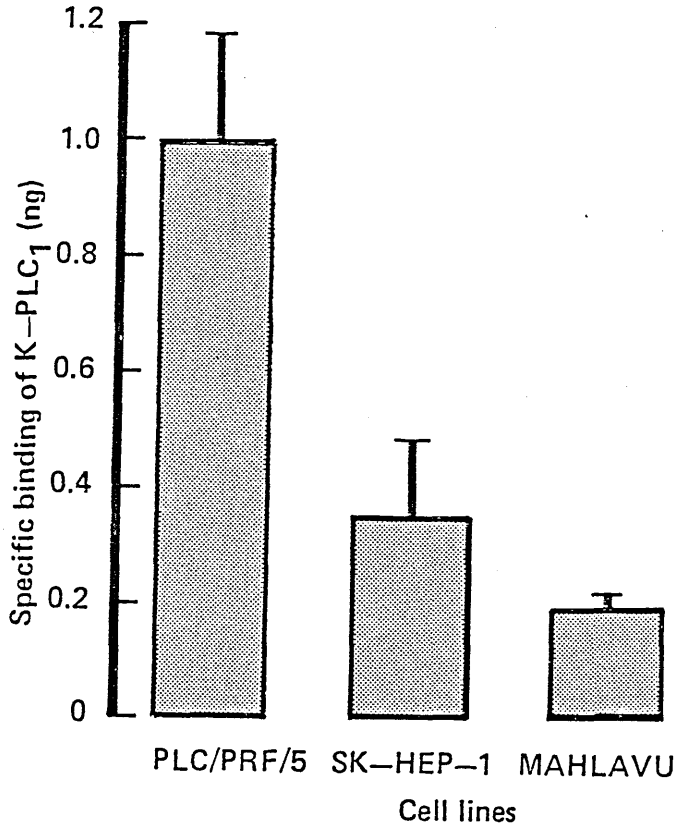

Figure 8 (v)

BINDING OF ^{125}I-K-PLC$_1$ TO THE PLC/PRF/5, SK-HEP-1 AND MAHLAVU CELL LINES. 50 µl of a 1:20 dilution of ^{125}I-K-PLC$_1$ (approx. 62 ng of protein) was added to 5 x 10^4 cells of each cell line for 2 hours. After centrifugation of the reaction mixture through silicone oil, the radioactivity bound to cell pellets was counted. Data represent the mean and standard deviation of 5 experiments each performed in triplicate.

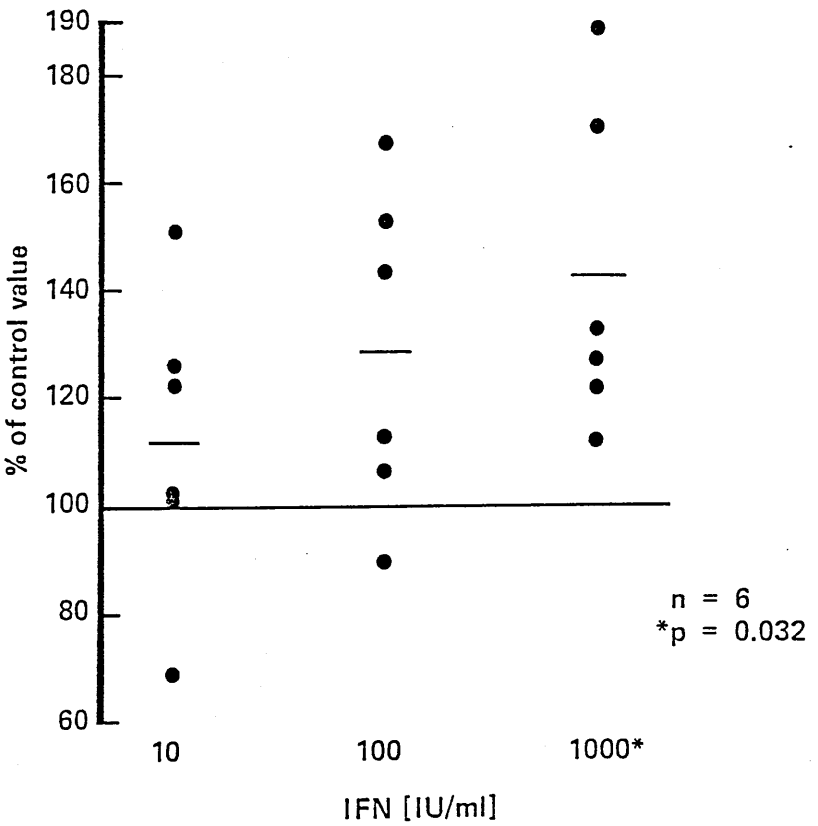

Figure 8 (vi)

THE EFFECT OF HUMAN LYMPHOBLASTOID IFN ON ^{125}I-K-PLC$_1$ BINDING TO PLC/PRF/5 CELLS. PLC/PRF/5 cells were incubated in medium containing IFN for 24 hours and then harvested and washed. 50 µl of a 1:20 dilution of ^{125}I-K-PLC$_1$ was added to 5 x 10^4 cells for 2 hours. Cells were pelleted by centrifugation through silicone oil and bound radioactivity counted. Results are expressed as a percentage of the binding observed with cells incubated in the absence of IFN. Each of the 6 experiments was performed in triplicate and bars represent mean values.

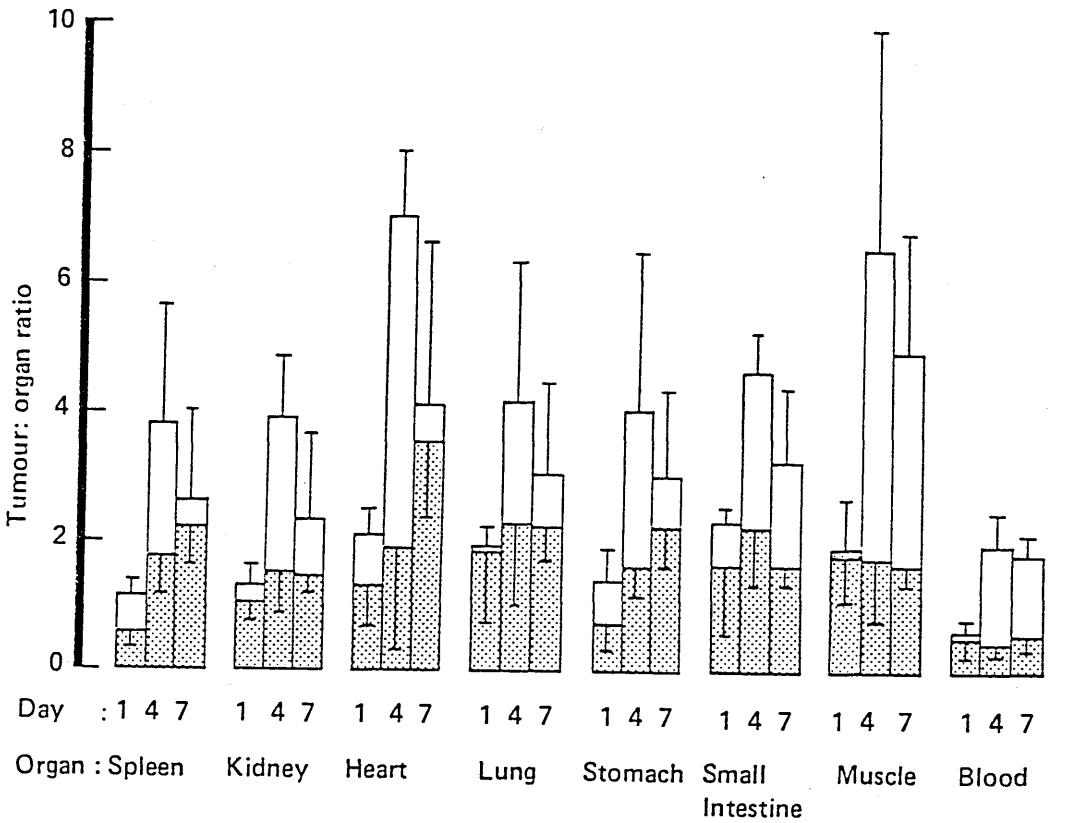

Figure 8 (vii)

TUMOUR:TISSUE RATIOS FOR ^{125}I-K-PLC$_1$ (OPEN BARS) AND ^{125}I-MOUSE IgG (SHADED BARS). Mice were injected intraperitoneally with 10 µg of either ^{125}I-K-PLC$_1$ (10-13 MBq) or ^{125}I-mouse IgG (12-14 MBq) and sacrificed at either 1, 4 or 7 days post injection. Radioactivity was counted in homogenised and extensively washed tissues and the results expressed as a ratio of counts/min/g in the tumours over counts/min/g in the tissues in question. Results are expressed as the mean and standard deviation of experiments in 5 animals.

With a view to targeting antibodies to lesions in the liver, tumour:liver ratios are obviously of importance. In this model system tumour:liver ratios for ^{125}I-K-PLC$_1$ were greater than those for ^{125}I-mouse IgG at all time points (Figure 8 (viii)). The differences observed between the antibodies was greatest on day four (ratio K-PLC$_1$ 4.4 $\pm$ 0.93, ratio mouse IgG 1.53 $\pm$ 0.60, mean $\pm$ SD, $p<0.05$, Wilcoxon's rank sum test), and remained significantly different at day seven. Tumour:blood ratios for ^{125}I-K-PLC$_1$ were much lower than any of the tumour:solid organ ratios (Figure 8 (vii)). At 24 hours the tumour:blood ratio was 0.57, indicating the presence of almost twice as much free circulating ^{125}I-K-PLC$_1$ as that bound to the tumour. By day four post injection the tumour:blood ratio had risen to 1.90 ($p<0.05$ vs ^{125}I-mouse IgG, Wilcoxon's rank sum test) and at day seven the ratio had declined slightly to 1.81.

Gamma camera pictures of an athymic mouse with an HCC xenograft taken at one and four days post injection with ^{125}I-K-PLC$_1$ are shown in Figure 8 (ix).

The sera of mice with large PLC/PRF/5-derived tumour xenografts did not competitively inhibit the binding of ^{125}I-K-PLC$_1$ to PLC/PRF/5 cells <u>in vitro</u>, suggesting that there is no significant secretion of K-PLC$_1$ antigen from tumour xenografts into mouse serum (data not shown).

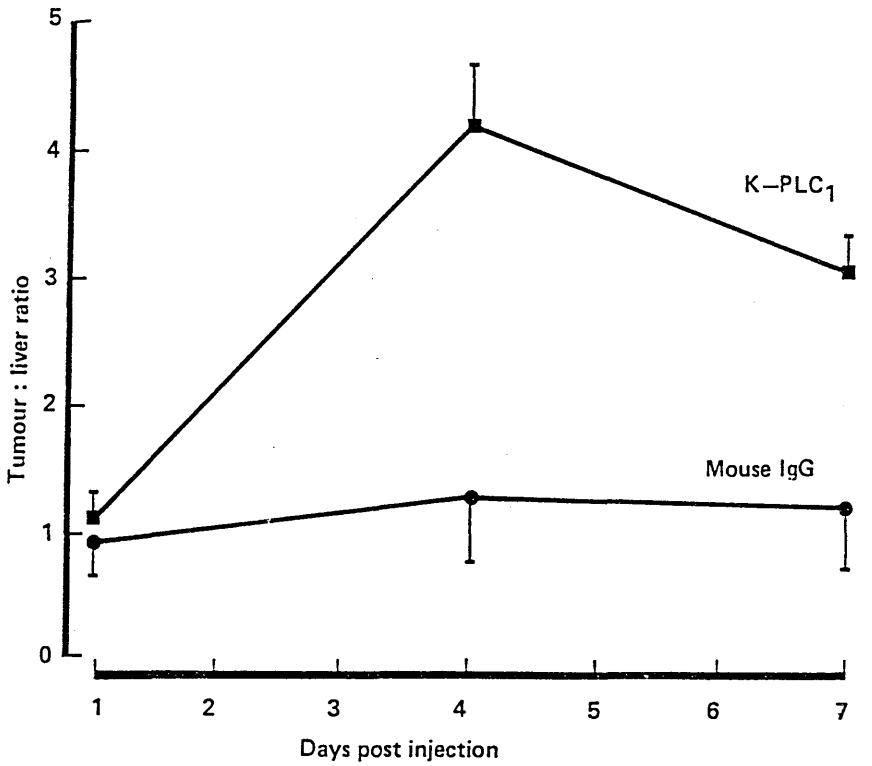

Figure 8 (viii)

TUMOUR:LIVER RATIOS FOR ^{125}I-K-PLC$_1$ AND ^{125}I-MOUSE IgG. Methodology is similar to that described in Figure 8 (vii). Each result is the mean $\pm$ standard deviation of the values obtained in 5 animals. The differences between the two antibodies is statistically significant on days 4 and 7 post injection.

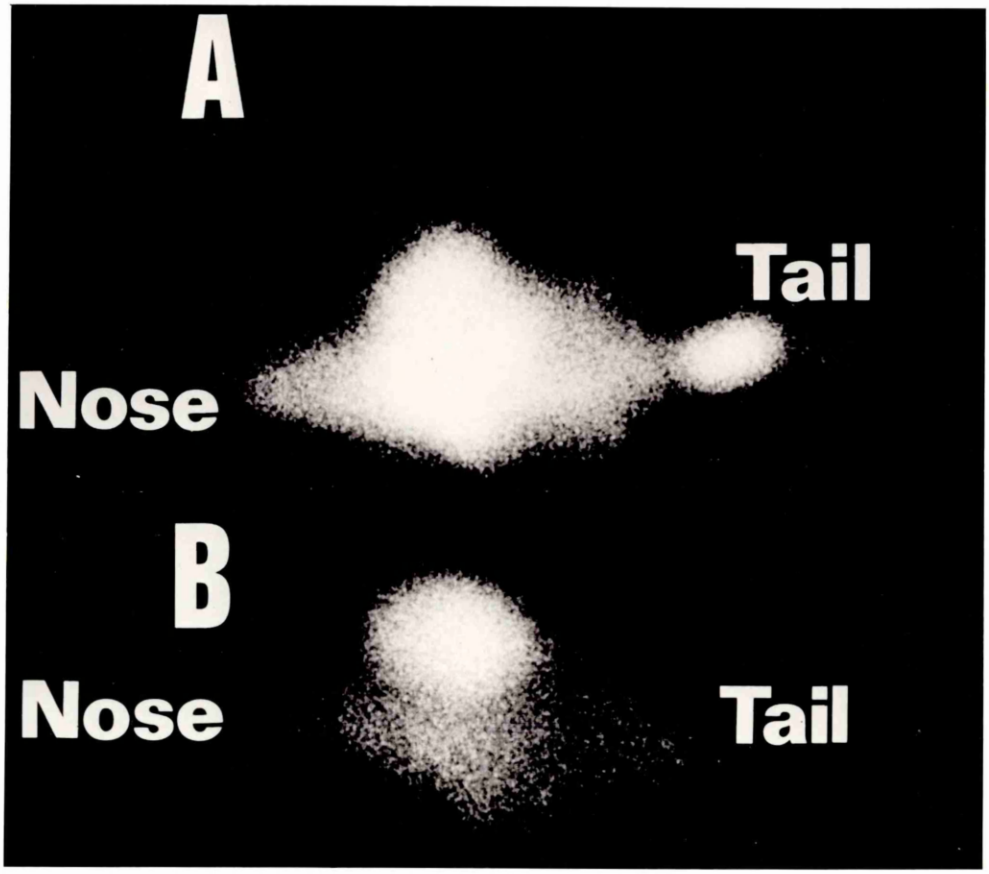

Figure 8 (ix)

GAMMA CAMERA PICTURES OF AN ATHYMIC MOUSE WITH A PLC/PRF/5-DERIVED TUMOUR XENOGRAFT GROWING OVER THE RIGHT SHOULDER.

Pictures were taken with a gamma camera fitted with a pin-hole collimator at 1 (A) and 4 (B) days following an IV injection of 10 µg of ^{125}I-K-PLC$_1$. The animal was sedated with intraperitoneal diazepam prior to examination and thyroid uptake of ^{125}I blocked by the prior addition of potassium iodide to the drinking water. On day 1 there is no obvious specific uptake of antibody by the tumour and the small area of increased uptake in the tail is due to tissue extravasation following tail vein injection. By day 4 there has been a decline in blood-pool radioactivity, allowing specific antibody uptake by the tumour to become clearly visible.

DISCUSSION

Wiedmann et al (in press) have shown that K-PLC$_1$ does not react with non-malignant hepatocytes, reacts only very weakly with vascular endothelium and some secretory epithelia, including bile duct epithelium, but reacts very strongly with a variety of carcinoma cell lines and tissue sections. As with almost all such antigen systems, the antigen recognised by K-PLC$_1$ is therefore tumour-associated and not tumour-specific.

This study has demonstrated abundant but heterogeneous K-PLC$_1$ antigen display by three HCC cell lines and by 10 of 11 HCC tumour specimens and it is therefore possible that K-PLC$_1$ may be of some use in immunocytohistochemistry, and enable the diagnosis of HCC to be made more easily from fine needle aspiration cytology specimens.

Secretion of K-PLC$_1$ antigen into the sera of mice with large HCC tumour xenografts was not detected in competitive inhibition experiments. While this precludes the use of this system as a potential serological tumour marker for HCC, this phenomenon may be advantageous in the setting of tumour imaging or drug targeting with monoclonal antibodies, as the reaction of secreted antigen with injected antibody in the circulation may theoretically stop antibody from reaching the tumour target, prolong antibody retention in serum, and lead to immune complex formation. These potential drawbacks, which may not be as great a problem in reality as would be expected theoretically (Dillman and Royston, 1984), are avoided by using antibodies such as K-PLC$_1$ which react with non-secreted tumour-associated antigens.

The tumour localisation experiments suggest that ^{125}I-K-PLC$_1$ binds preferentially to HCC tumour xenografts rather than to other solid tissues and that tumour:non-tumour antibody ratios are greatest four days after antibody injection, a time period similar to that noted by other authors (Moshakis et al, 1981; Epenetos et al, 1982). Tumour:liver ratios were however rather lower than hoped for, and blood-pool radioactivity was high throughout the study period. Methods which can increase target antigen display by the tumour or enhance unbound antibody clearance from the circulation should improve tumour:non-tumour antibody ratios and potentially enhance the value of monoclonal antibodies as immunodiagnostic or therapeutic agents.

Interferons may increase tumour-associated antigen display (Greiner et al, 1984) and therefore be of some benefit in this regard. In vitro, human lymphoblastoid IFN increased K-PLC$_1$ antigen display by PLC/PRF/5 cells and further studies are required to determine whether IFNs can affect K-PLC$_1$ antigen display in vivo. Clearance of circulating unbound radiolabelled antibody can be enhanced by the administration of a liposomally entrapped second antibody against the first antibody (Begent et al, 1982). While this technique could prove advantageous for targeting to peripheral tumours, the removal of liposomes by the reticuloendothelial system could well complicate the targeting of lesions within the liver. Of greater application with respect to liver lesions may be the use of F(ab) or F(ab)$_2$ antibody fragments. Antibody is removed rapidly from the circulation after the elimination of its Fc piece (Hopf et al, 1976) and several studies have now demonstrated improved tumour targeting when F(ab) (Larson et al, 1983; Carlson et al, 1985) or F(ab)$_2$ fragments (Houston et al, 1980; Bate et al, 1980; Wahl et al, 1983) are used in place of intact antibody.

Despite the fact that a variety of tumours can be successfully imaged by radiolabelled monoclonal antibodies (for review see Baldwin and Byers, 1985) it is unlikely that this technique will approach the diagnostic accuracy of currently available imaging modalities in the near future. Probably the most important use of anti-tumour monoclonal antibodies in the future will be to specifically target either drugs, radioisotopes or cellular toxins to tumours and therefore provide a highly selective and hopefully non-toxic means of killing tumours and their metastases. Such therapy is at an early stage of development, and of the many potential problems of antibody therapy (Embleton, 1987), the problems of antigenic heterogeneity and the antigenicity of murine monoclonal antibodies in man particularly stand out.

Antigenic heterogeneity, as demonstrated by the K-PLC_1 antigen in this study, is common among tumour-associated antigens (Edwards, 1985). It has previously been shown that the anti-tumour response to passively administered monoclonal antibody is dependent on the level of target antigen display by the tumour in question, and it has been suggested that a single passively administered monoclonal antibody is unlikely to eradicate solid tumours completely (Capone et al, 1984). To overcome this potential problem it may be necessary to administer antibody combinations (for example K-PLC_1 plus other anti-HCC (Shouval et al, 1985; Carlson et al, 1985; Hu et al, 1986) or anti-AFP antibodies (Tsukada et al, 1985)) in order to reach all the cells in a given tumour, or alternatively a single monoclonal antibody linked to a high energy β-radiation emitter such as ^{131}Iodine may be able to destroy cells bearing the target antigen plus all cells around

it for many cell diameters. Similarly,the problem of the antigenicity of murine antibodies, which may lead to allergic reactions and to reduced antibody effectiveness, may not be as great in practice as expected in theory (Schroff et al, 1985), and may be overcome in the future by the development of human monoclonal antibodies (Sikora, 1984).

Encouraged by the fact that the problems of monoclonal antibody therapy in man may be soluble in the near future, and by recent work demonstrating that the development of HCC tumour xenografts in athymic mice can be inhibited by monoclonal anti-HBs (Shouval et al, 1982), and that the growth of established HCC xenografts can be inhibited in these animals either by monoclonal anti-HBs linked to the plant toxin ricin (Oladapo et al, 1984) or by a drug such as daunorubicin conjugated to anti-AFP antibodies (Tsukada et al, 1985), the immunotherapeutic potential of native, isotope and ricin-conjugated K-PLC_1 is currently being evaluated in the athymic mouse model.

CHAPTER NINE

RECOMMENDATIONS FOR FURTHER STUDIES AND CONCLUDING REMARKS

Like most pieces of research, this thesis has perhaps raised more questions than it has answered. In the light of my findings I have listed below further studies which I think are required.

FURTHER STUDIES

Chapter 1

HCC, as it is seen in the United Kingdom, is clearly diagnosed late in its natural history, and curative resection is rarely possible. It remains to be determined whether prospective screening of the British cirrhotic population, using a combination of repeated serum AFP measurements and real-time hepatic ultrasonography, is effective in diagnosing asymptomatic HCC patients with small tumours at a stage when curative surgery is possible.

Liver cell dysplasia was frequently observed in British HCC patients, and in some cases this preceeded tumour development by many years. It would be of interest to determine the exact frequency of liver cell dysplasia in the British cirrhotic population and to prospectively follow such a group to determine whether patients with dysplasia are at greater risk of tumour development than those without.

Further studies are needed to more accurately determine the relationship between HBV infection and HCC development. The exact frequency with which integrated HBV-DNA sequences can be detected in the tumours of serum HBsAg positive and negative patients requires to be determined more accurately in the British HCC population, as so far

it has been possible to study only a relatively small number of patients. The molecular mechanisms whereby HBV-DNA integration may eventually result in cellular transformation remain to be fully elucidated.

Chapters 2 and 3

Much of the literature on HCC treatment is unsatisfactory and many questions remain unanswered. As liver transplantation becomes more widely available, its role in the treatment of patients with non-resectable lesions requires to be determined. Controlled clinical trials are needed to determine whether intra-arterial drug administration is better than simple intravenous treatment with the same agent. Similarly, controlled trials are needed to examine the role of "lipiodolised" drug treatment in HCC, and the efficacy of several combinations of therapeutic modalities could be usefully examined.

A phase III study is needed to compare doxorubicin and mitozantrone in the treatment of inoperable HCC patients.

Chapters 5 and 6

A comparison of the effects of each IFN class (α , β and γ) on the in vitro and in vivo growth of HCC cell lines would be of interest, as would studies comparing their effects on tumour 2,5A synthetase activity and HLA class I glycoprotein display. Similarly the effects of a particular IFN class on the growth of several different HCC cell lines should be studied, in order to determine whether there are any important differences in IFN sensitivity between cell lines.

Extensive phase I and phase II studies of each IFN class need to be undertaken in HCC patients before the role of IFN in HCC treatment can be determined with any certainty. A study of the effects of chronic low dose IFN therapy on immunological parameters in HCC patients would be of some interest, and further studies are needed to determine the pathogenesis of the reduced NK cell activity in HCC patients.

Chapter 7

The frequency with which nuclear androgen receptors may be found in samples of normal adult liver, cirrhotic liver, and HCC tissue remains to be accurately determined. The biological significance of the presence of nuclear AR in these sites requires elucidation. If *in vitro* studies suggest that the low concentrations of AR present in HCC tumour xenografts are sufficient to promote molecular effects such as the transcription of specific messenger RNA sequences, then further studies in large numbers of xenografted animals, in which the hormonal environment is manipulated medically, would be of interest.

Chapter 8

This chapter perhaps raises more questions than any of the others. The value of K-PLC_1 as an immunocytochemical reagent for the diagnosis of HCC from fine needle aspiration cytology specimens should be determined. Immunoscintigraphic studies with radiolabelled K-PLC_1 are currently under way in HCC patients and their results should indicate whether K-PLC_1 is of value in the visualisation of tumours and their metastases.

In vitro studies are required to determine the effects of each IFN class on K-PLC_1 expression by HCC cell lines. The effect of *in vivo*

IFN administration of K-PLC$_1$ antigen display by HCC tumour xenografts should be examined, and, as pilot studies of IFN therapy in HCC patients are now underway, it may be possible to examine this phenomenon in liver biopsy specimens taken before and during IFN treatment.

The immunotherapeutic potential of native, isotopically labelled, and ricin-conjugated K-PLC$_1$ is currently being explored in athymic mice with HCC tumour xenografts.

CONCLUSIONS

While the immediate outlook for HCC patients undoubtedly remains gloomy, there are now several rays of hope on the horizon.

The development of effective immunisation schedules against perinatally acquired HBV infection brings the exciting prospect of tumour prevention through vaccination a step closer to reality in areas of high HCC incidence. The application of such schedules must become a public health priority in these regions, and the development of cheap genetically engineered and chemically synthesised HBV vaccines will hopefully bring the cost of immunisation within the reach of the many Third World countries involved. As with all disease, prevention is better than cure.

In areas of low HCC incidence, where HBV infection is much less often implicated as an aetiological factor in hepatic carcinogenesis, there is little prospect of significantly reducing HCC incidence by vaccination against the HBV. In these areas in particular, screening

methods must be developed which allow the detection of small resectable tumours in asymptomatic patients. The need for such techniques is clearly emphasised by the finding in British HCC patients of a median duration of survival of only seven weeks from diagnosis (Chapter 1). The value of screening patients with chronic liver disease by a combination of repeated serum AFP estimation and real-time hepatic ultrasonography has been established in the Asian population, and this method should now be evaluated as a matter of some urgency in other regions. It should be remembered however, that at least in the United Kingdom, a significant proportion of patients with chronic liver disease present de novo with HCC and will escape screening (Chapter 1).

There will therefore continue to be an urgent need for new and more efficacious medical therapies for patients with inoperable HCC. Progress in this field has been much less dramatic than that seen in other areas. This seems likely to change in the near future however, as exciting new treatment modalities, several of which act wholly or partly by modifying host biological responses, are evaluated. In this thesis I have explored three areas of therapeutic opportunity. Preliminary experiments involving androgenic manipulation did not prove as therapeutically promising as had been hoped. However both interferon therapy and immunotherapy with monoclonal antibodies offer real hope for future treatment. These materials are now available in relative abundance, and the next few years will hopefully see them advance from the laboratory to extensive evaluation in the clinical setting.

APPENDIX

The findings of this thesis have been presented at national and international meetings in Europe and the USA and published in abstract form as listed below:

J Hepatol 1984; 1 (Suppl 1): A96

Gut 1984; 25: A1131

Gut 1985; 26: A561

Hepatology 1985; 5: A78

Gut 1986; 27: A616

Gut 1986; 27: A1265

Scot Med J 1986; 31: 263-264

Much of the work has either been published in full or is currently in press, and the remainder has been submitted for publication. Those papers already published or in press are listed below and reprints may be found in the inside back pocket of the thesis.

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