Thesis scanned from best copy available: may contain faint or blurred text, and/or cropped or missing pages.

**Digitisation Notes:**

- Page 297 missing in original
MECHANISMS OF CANCER CACHEXIA

STEPHEN J WIGMORE
BSc (hons), MB BS (Lond), FRCSED

Submitted for the degree
of
Doctor of Medicine
to
University of Edinburgh
1997
For Forbes
DECLARATION

This thesis was composed solely by the author. The work presented in the thesis was performed by the author while in post in South-East Scotland except where stated. Due acknowledgement of this assistance is given in the relevant sections. The work presented in this thesis has not been submitted in candidature for any other degree, diploma or professional qualification.

Stephen J Wigmore
ABSTRACT

Cachexia is a common problem affecting patients with gastrointestinal cancer and is associated with considerable morbidity and mortality. The purpose of this thesis was to investigate the mechanisms of cancer cachexia and use the information so obtained to attempt to develop effective treatment strategies using pancreatic cancer as a model of cancer cachexia.

Longitudinal nutritional assessment in a group of untreated patients with pancreatic cancer demonstrated that weight loss with depletion of lean tissue and subcutaneous fat stores was an inexorable process up to death. Analysis of energy balance in a similar cohort of patients demonstrated increased resting energy expenditure (REE) compared with predicted values and reduced nutritional intake and showed that patients who had an elevated acute phase protein response (APPR) had the greatest energy deficits.

An investigation was undertaken into the aetiology of the APPR in cancer. Peripheral blood mononuclear cell (PBMC) interleukin-6 (IL-6) and tumour necrosis factor (TNF) production were elevated compared with controls and the supernatants from these cells stimulated a greater intensity of APPR by isolated human hepatocytes compared with supernatants from control PBMC. Pre-treatment of PBMC with IL-4 reduced the ability of PBMC from cancer patients to produce IL-6 and TNF and reduced the potential of these supernatants to elicit an APPR in vitro. Human pancreatic cancer cell lines were found to secrete IL-6 and/or IL-8 constitutively. During investigations into the effects of pancreatic cancer cell line supernatants on APP production by hepatocytes it was observed that IL-8 could mediate APP production in vitro. Serum concentrations of both IL-6 and IL-8 were elevated in pancreatic cancer patients and IL-8 correlated with serum CRP concentration.

Having established the potential for increased elaboration of pro-inflammatory cytokines both by PBMC and by tumour cell lines and the ability of these cytokines to
stimulate an acute phase response in vitro, a number of studies were undertaken using drugs with the potential to modify the cytokine-hepatocyte axis in vivo. The first study used the anti-inflammatory drug ibuprofen to establish whether reducing the APPR in vivo would reduce REE. Patients receiving ibuprofen experienced significant reduction in serum CRP concentration and REE compared with placebo. A further study was undertaken combining longer term administration of ibuprofen with the appetite stimulant megestrol acetate. This study demonstrated significant weight gain in patients receiving ibuprofen and improvement in quality of life indices compared with placebo.

Polyunsaturated fatty acids notably eicosapentaenoic acid (EPA) have been shown to reduce cytokine production by inflammatory cells. A study was undertaken to investigate the effect of oral supplementation with a crude fish oil preparation containing 18% EPA on the progress of cachexia. Three quarters of patients who received fish oil became weight-stable or gained a small amount of weight. A further study undertaken using a larger effective dose of EPA in a high purity preparation demonstrated a similar anti-cachectic effect to fish oil. This confirmed that EPA was an anti-cachectic agent but that no dose response effect was evident over the dose range studied. Administration of EPA was shown to result in reduced production of IL-6 by PBMC and reduced potential of PBMC supernatants to elicit an APPR by isolated human hepatocytes. The clinical effect of EPA on the APPR was however variable.

These investigations highlight the importance of the cytokine-hepatocyte axis and the APPR in cancer cachexia. Clinical trials of drugs directed at reducing cytokine concentrations and the APPR have shown early promise and are worthy of further evaluation in the management of cancer cachexia.
ACKNOWLEDGEMENTS

The work presented in this thesis was performed over a three year period in the University Department of Surgery at the Royal Infirmary of Edinburgh. I am most grateful to my supervisors Dr Jim Ross and Mr Ken Fearon for their constant support, advice, tolerance and humour. I am also grateful to Prof Sir David Carter for his guidance and support and to Mr O.J. Garden who supplied liver tissue for the isolation of hepatocytes.

I am extremely grateful for the excellent technical support from Jean Maingay without whom much of the laboratory work would not have been possible. I am most grateful to Paul Lai for isolating IL-8 mRNA from pancreatic cancer cells. I thank also Katherine Sangster, Ian Ansell and Dr Tony Hawkins for their advice and assistance.

I am particularly grateful to Claire Plester (Wright) for her help in assessing nutritional indices in many of the patients and to Matthew Barber for completing the follow up on patients in the high purity EPA trial. I thank also my contemporary research fellows Andrew de Beaux, Tracey Gillies, Paul Lai and Stuart Falconer for their friendship and Mike O'Riordain for helping me get the hepatocyte perfusion up and running.

I thank Prof Mike Tisdale (Aston University) for his cheerful interest and advice and for performing plasma and erythrocyte membrane fatty acid analysis in the EPA trials.

Thanks also to Dr Donny McMillan (University of Glasgow) for his help in running the megestrol acetate / ibuprofen trial.

I am grateful to the following for their financial support:

The Smith & Nephew Foundation
The University of Edinburgh Wilkie Scholarship Committee
The University of Edinburgh Cancer Research Fund
Sir Stanley and Lady Davidson Research Bequest
The Royal College of Surgeons of Edinburgh
The Leverhume Trust
Scotia Pharmaceuticals
Cancer Research Campaign.

Finally I would like to thank all of my family for their unstinting interest and encouragement and especially my wife Lynne for "being there".
ABBREVIATIONS

AA=Arachidonic acid
ACT = Alpha 1 antichymotrypsin
AGP = Alpha 1 acid glycoprotein
AMC=Arm muscle circumference
APP= Acute phase protein(s)
APPR = Acute phase protein response
BIA = Bioelectrical impedance analysis
BMI=Body mass index
BSA=Bovine serum albumin
CRP= C-reactive protein
DHA=Docosahexaenoic acid
ELISA=enzyme-linked immunosorbent assay
EPA=Eicosapentaenoic acid
FCS=Foetal calf serum
GLA=Gamma linolenic acid
IL=Interleukin
MFBIA=Multifrequency bioelectrical impedance analysis
MUAC=Mid upper arm muscle circumference
PBMC=Peripheral blood mononuclear cell
PREAL= Prealbumin
REE= Resting energy expenditure
SDS PAGE= Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEE=Total energy expenditure
TMB=5,5',3,3' tetra methyl benzidine
TNF= Tumour necrosis factor
TRF= Transferrin
TSF=Triceps skinfold thickness
INDEX

INTRODUCTION 1
Cachexia-the scale of the problem 2
Clinical significance of cachexia in the cancer host 3
Mechanisms of cancer cachexia 4
Carbohydrate metabolism 4
Lipid metabolism 5
Protein metabolism 6
THE ANOREXIA-CACHEXIA SYNDROME 7
Body composition in cancer cachexia 7
The concept of energy balance 8
Factors leading to reduced energy intake 11
The cytokine/acute phase protein response in cancer cachexia 12
Isolated hepatocyte cultures 15
The acute phase protein response and skeletal muscle protein 16
Production of pro-inflammatory cytokines by peripheral blood mononuclear cells 17
Production of cytokines by tumour cell lines 18
Direct cachectic factors 19
Carcinoma of the pancreas as a model of cancer cachexia 20
Trials of intervention in cancer cachexia 21
Supplementation of intake 21
Specific anti-cachectic strategies 23
Corticosteroids 23
Hydrazine sulphate 23
Tetrahydrocannabinol 24
Insulin infusions 24
Cyproheptadine 24
Chapter 2

FREQUENTLY QUOTED METHODS 32

Isolation of human hepatocytes 33
Materials 33
Perfusion solutions 33
Apparatus set up 34
Collection of specimens 35
Preparation of Specimens 35
Perfusion procedure 35
Purification of hepatocytes 37
Preparation of tissue culture plates 38
Plating of hepatocytes 39
Hepatocyte culture 39
Hepatocyte viability 39
Comment 40

ISOLATION OF PERIPHERAL BLOOD MOONUCLEAR CELLS 46

Stimulation of PBMC with lipopolysaccharide 46
Stimulation of PBMC with phytohaemagglutinin 47
Measurement of $^3$H thymidine uptake 48

CYTOKINE AND ACUTE PHASE PROTEIN ASSAYS 48
Measurement of IL-8, IL-6 and TNF in cell culture supernatants 48
Measurement of acute phase proteins in hepatocyte supernatants 50
Measurement of C-reactive protein concentration in sera 51
CULTURE OF HUMAN PANCREATIC CANCER CELL LINES 54
ANALYSIS OF PLASMA AND ERYTHROCYTE MEMBRANE
FATTY ACID COMPOSITION 55
NUTRITIONAL ASSESSMENT TECHNIQUES 56
Anthropometry 56
Body composition analysis 56
Resting energy expenditure 56
Assessment of nutritional intake 57
Predictive equations for resting energy expenditure 57
QUALITY OF LIFE ASSESSMENT 62

Chapter 3
NUTRITIONAL STATUS OF PATIENTS WITH PANCREATIC CANCER
Introduction 64
Patients and methods 65
Results 66
Patient characteristics 66
Weight loss 67
Body mass index 68
Arm muscle circumference 68
Triceps skinfold thickness 70
Body composition 70
Discussion 72

Chapter 4
# NUTRITIONAL INTAKES AND THE CONTRIBUTION OF ANOREXIA AND HYPERMETABOLISM TO ENERGY DEFICIT IN PATIENTS WITH PANCREATIC CANCER

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>77</td>
</tr>
<tr>
<td>Patients and methods</td>
<td>78</td>
</tr>
<tr>
<td>Results</td>
<td>80</td>
</tr>
<tr>
<td>Patient characteristics</td>
<td>80</td>
</tr>
<tr>
<td>Nutritional intakes</td>
<td>80</td>
</tr>
<tr>
<td>Energy expenditure</td>
<td>80</td>
</tr>
<tr>
<td>Calculation of energy deficits</td>
<td>87</td>
</tr>
<tr>
<td>Serum C-reactive protein and albumin concentrations</td>
<td>87</td>
</tr>
<tr>
<td>Energy balance in patients with pancreatic cancer in relation to the presence of serum acute phase protein concentration</td>
<td>87</td>
</tr>
<tr>
<td>Associations between the APPR and energy intake and resting energy expenditure</td>
<td>88</td>
</tr>
<tr>
<td>Discussion</td>
<td>92</td>
</tr>
</tbody>
</table>

## Chapter 5

**PRO-INFLAMMATORY CYTOKINE PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS ISOLATED FROM PATIENTS WITH CANCER, MULTIPLE ORGAN FAILURE AND HEALTHY CONTROLS AND THE ACUTE PHASE RESPONSE IN VITRO.**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>95</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>97</td>
</tr>
<tr>
<td>Results</td>
<td>99</td>
</tr>
<tr>
<td>Patient characteristics</td>
<td>99</td>
</tr>
<tr>
<td>IL-6 and TNF release by PBMC</td>
<td>100</td>
</tr>
<tr>
<td>Hepatic acute phase protein synthesis</td>
<td>104</td>
</tr>
<tr>
<td>Positive acute phase protein production</td>
<td>105</td>
</tr>
</tbody>
</table>
### Chapter 7
SERUM INTERLEUKIN-8 AND INTERLEUKIN-6 CONCENTRATIONS IN PATIENTS WITH PANCREATIC CANCER

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>166</td>
</tr>
<tr>
<td>Patient and methods</td>
<td>166</td>
</tr>
<tr>
<td>Results</td>
<td>168</td>
</tr>
<tr>
<td>Patient characteristics</td>
<td>168</td>
</tr>
<tr>
<td>Serum IL-6, IL-8 and CRP concentrations</td>
<td>168</td>
</tr>
<tr>
<td>Cytokines and the acute phase response</td>
<td>172</td>
</tr>
<tr>
<td>Discussion</td>
<td>177</td>
</tr>
</tbody>
</table>

### Chapter 8
EFFECT OF IBUPROFEN ON ENERGY EXPENDITURE AND ACUTE PHASE PROTEIN PRODUCTION IN PANCREATIC CANCER PATIENTS COMPARED WITH PLACEBO

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>181</td>
</tr>
<tr>
<td>Patients and Methods</td>
<td>182</td>
</tr>
<tr>
<td>Results</td>
<td>185</td>
</tr>
<tr>
<td>Patient characteristics</td>
<td>185</td>
</tr>
<tr>
<td>Differences in nutritional indices between cancer and non-cancer patients</td>
<td>185</td>
</tr>
<tr>
<td>Differences in nutritional indices in patients with pancreatic cancer receiving ibuprofen and placebo</td>
<td>185</td>
</tr>
<tr>
<td>Resting energy expenditure</td>
<td>187</td>
</tr>
<tr>
<td>Serum C-reactive protein concentrations</td>
<td>188</td>
</tr>
<tr>
<td>Discussion</td>
<td>191</td>
</tr>
</tbody>
</table>

### Chapter 9
RANDOMISED DOUBLE BLIND, PLACEBO-CONTROLLED TRIAL OF THE EFFECT OF MEGESTROL ACETATE AND IBUPROFEN ON THE PROGRESS OF CACHEXIA IN PATIENTS WITH PANCREATIC CANCER

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>195</td>
</tr>
<tr>
<td>Chapter 10</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>EFFECT OF ORAL SUPPLEMENTATION WITH FISH OIL IN WEIGHT LOSING</td>
<td>212</td>
</tr>
<tr>
<td>PATIENTS WITH PANCREATIC CANCER</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>212</td>
</tr>
<tr>
<td>Patients and methods</td>
<td>215</td>
</tr>
<tr>
<td>Results</td>
<td>217</td>
</tr>
<tr>
<td>Patient characteristics</td>
<td>217</td>
</tr>
<tr>
<td>Tolerance</td>
<td>218</td>
</tr>
<tr>
<td>Weight changes</td>
<td>218</td>
</tr>
<tr>
<td>Comparison between weight changes following fish oil supplementation</td>
<td>218</td>
</tr>
<tr>
<td>and supplementation with gammalinolenic acid (GLA)</td>
<td></td>
</tr>
<tr>
<td>Acute phase protein response</td>
<td>222</td>
</tr>
<tr>
<td>Fatty acid composition</td>
<td>225</td>
</tr>
<tr>
<td>Discussion</td>
<td>230</td>
</tr>
<tr>
<td>Chapter 11</td>
<td></td>
</tr>
</tbody>
</table>
EFFECT OF HIGH PURITY ORAL EICOSAPENTAENOIC ACID ON THE PROGRESS OF CACHEXIA IN WEIGHT-LOSING PATIENTS WITH PANCREATIC CANCER

Introduction 235
Patients and methods 236
In vitro methods 239
Results 241
Patient characteristics 241
Tolerance/toxicity 242
Changes in body weight 242
Anthropometry and body composition analysis 245
Acute phase protein response 245
Nutritional intake 247
Fatty acid analysis 247
Performance status 247
Survival 249
Results of in vitro experiments 251
Effect of EPA on cancer patients serum CRP and IL-6 levels on isolated PBMC IL-6 production rates 251
Effect of recombinant IL-6 and IL-6 neutralising antibody on CRP production by isolated human hepatocytes 253
Effect of neutralising antibody to IL-6 on the ability of PBMC supernatants to stimulate CRP production by isolated hepatocytes. 254
Effect of EPA on the ability of PBMC supernatants from cancer patients to stimulate production of other acute phase proteins by isolated human hepatocytes. 258
Effect of EPA on incorporation of ³H thymidine by lymphocytes in the presence and absence of phytohaemagglutinin. 258
Discussion 266
GENERAL DISCUSSION 271
References 282
Appendix 1 List of presentations relating to submitted work 298
Appendix 2 List of publications relating to submitted work 301
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Body composition in healthy controls and cancer patients</td>
<td>8</td>
</tr>
<tr>
<td>1.2</td>
<td>Components of energy expenditure</td>
<td>9</td>
</tr>
<tr>
<td>1.3</td>
<td>Energy balance in patients with cancer</td>
<td>10</td>
</tr>
<tr>
<td>1.4</td>
<td>Cytokine regulation of the acute phase protein response</td>
<td>18</td>
</tr>
<tr>
<td>2.1a</td>
<td>Hepatocyte perfusion apparatus</td>
<td>37</td>
</tr>
<tr>
<td>2.1</td>
<td>Hepatocyte isolation technique-priming cycle</td>
<td>43</td>
</tr>
<tr>
<td>2.2</td>
<td>Hepatocyte isolation technique-perfusion cycle</td>
<td>44</td>
</tr>
<tr>
<td>2.3</td>
<td>Hepatocyte isolation technique-recirculation loop</td>
<td>45</td>
</tr>
<tr>
<td>2.4</td>
<td>Correlation between measurement of CRP by ELISA and laboratory</td>
<td>53</td>
</tr>
<tr>
<td>2.5</td>
<td>Correlation between recorded REE and Harris-Benedict prediction</td>
<td>60</td>
</tr>
<tr>
<td>2.6</td>
<td>Correlation between recorded REE and Scholfield prediction</td>
<td>61</td>
</tr>
<tr>
<td>3.1</td>
<td>Weight loss in patients with pancreatic cancer</td>
<td>69</td>
</tr>
<tr>
<td>4.1</td>
<td>Nutrient intakes in pancreatic cancer patients</td>
<td>82</td>
</tr>
<tr>
<td>4.2</td>
<td>Vitamin intakes in pancreatic cancer patients</td>
<td>83</td>
</tr>
<tr>
<td>4.3</td>
<td>Mineral intakes in pancreatic cancer patients</td>
<td>84</td>
</tr>
<tr>
<td>4.4</td>
<td>Amino acid intakes in pancreatic cancer patients</td>
<td>85</td>
</tr>
<tr>
<td>4.5</td>
<td>Fatty acid intakes in pancreatic cancer patients</td>
<td>86</td>
</tr>
<tr>
<td>4.6</td>
<td>Correlation between CRP and anorexia</td>
<td>89</td>
</tr>
<tr>
<td>4.7</td>
<td>Correlation between CRP and hypermetabolism</td>
<td>90</td>
</tr>
<tr>
<td>4.8</td>
<td>Distribution of energy deficit in relation to the APPR</td>
<td>91</td>
</tr>
<tr>
<td>5.1a</td>
<td>Effect of IL-4 on ACT production by hepatocytes</td>
<td>106</td>
</tr>
<tr>
<td>5.2a</td>
<td>Effect of IL-2 on ACT production by hepatocytes</td>
<td>107</td>
</tr>
<tr>
<td>5.1b</td>
<td>Effect of IL-4 on acid 1 glycoprotein production by hepatocytes</td>
<td>110</td>
</tr>
<tr>
<td>5.2b</td>
<td>Effect of IL-2 on acid 1 glycoprotein production by hepatocytes</td>
<td>111</td>
</tr>
<tr>
<td>5.1c</td>
<td>Effect of IL-4 on CRP production by hepatocytes</td>
<td>112</td>
</tr>
<tr>
<td>5.2c</td>
<td>Effect of IL-2 on CRP production by hepatocytes</td>
<td>113</td>
</tr>
<tr>
<td>5.1d</td>
<td>Effect of IL-4 on transferrin production by hepatocytes</td>
<td>115</td>
</tr>
</tbody>
</table>
5.2d Effect of IL-2 on transferrin production by hepatocytes 116
5.1e Effect of IL-4 on prealbumin production by hepatocytes 118
5.2e Effect of IL-2 on prealbumin production by hepatocytes 119
6.1 Production of IL-8 by human pancreatic cancer cell lines 134
6.2 Production of IL-6 by human pancreatic cancer cell lines 136
6.3 RF plot of IL-8 band from SDS PAGE analysis 138
6.4 Western blot of pancreatic cancer cell-derived IL-8 139
6.5 RT PCR of pancreatic cancer cell-derived IL-8 mRNA 140
6.6 Effect of IL-1, TNF, IL-4 on IL-8 production by MiaPaCa2 cells 142
6.7 Effect of IL-1, TNF, IL-4 on IL-8 production by CFPAC cells 144
6.8 Stimulation of CRP production by pancreatic cell supernatants 146
6.9 Effect of anti-IL-8 antibody on CRP production 147
6.10 Effect of rhIL-8 on CRP production by isolated hepatocytes 149
6.11 Effect of rhIL-8 on ACT production by isolated hepatocytes 150
6.12 Effect of rhIL-8 on AGP production by isolated hepatocytes 151
6.13 Effect of rhIL-8 on transferrin production by isolated hepatocytes 152
6.14 Effect of rhIL-8 on prealbumin production by isolated hepatocytes 153
6.15a Effect of rhIL-8 on AGP production by HepG2 cells 155
6.15b Effect of rhIL-8 and rhIL-6 on AGP production by HepG2 cells 156
6.16a Effect of rhIL-8 on haptoglobin production by HepG2 cells 155
6.16b Effect of rhIL-8 and rhIL-6 on haptoglobin production by HepG2 cells 156
6.17a Effect of rhIL-8 on transferrin production by HepG2 cells 155
6.17b Effect of rhIL-8 and rhIL-6 on transferrin production by HepG2 cells 156
7.1 Serum concentrations of IL-8 and IL-6 in pancreatic cancer patients 170
7.2 Serum concentrations of CRP in pancreatic cancer patients 171
7.3 Correlation between serum IL-6 and IL-8 174
7.4 Correlation between serum CRP and IL-8 175
7.5 Correlation between serum IL-6 and CRP 176
8.1 Effect of ibuprofen on serum CRP concentrations 190
9.1 Appetite and intake scores of patients receiving megestrol/ibuprofen
9.2 Weight change in patients receiving megestrol/ibuprofen
9.3 Survival in patients receiving megestrol/ibuprofen
10.1 Weight changes in patients receiving fish oil
10.2 Weight changes in patients receiving gamma linolenic acid
10.3 Correlation between weight change and REE in patients receiving fish oil
10.4 Plasma fatty acid composition in patients receiving fish oil
10.5 Erythrocyte membrane fatty acid composition
10.6 % plasma EPA concentration versus dosage of fish oil
10.7 Weight change in relation to dosage of fish oil
11.1 Changes in weight in patients receiving EPA
11.2 Changes in serum CRP in patients receiving EPA
11.3 Survival duration in patients receiving EPA
11.4 Serum CRP and IL-6 in subgroup of patients receiving EPA
11.5 PBMC IL-6 production in subgroup of patients receiving EPA
11.6 Effect of IL-6 and anti IL-6 antibody on CRP production
11.7 CRP production by hepatocytes treated with PBMC supernatants
11.8 Effect of anti-IL-6 antibody on CRP production
11.9 Effect of EPA on ACT production by hepatocytes
11.10 Effect of EPA on AGP production by hepatocytes
11.11 Effect of EPA on prealbumin production by hepatocytes
11.12 Effect of EPA on transferrin production by hepatocytes
11.13 Effect of EPA on T-cell blastogenesis
11.14 Change in plasma fatty acid composition following EPA
Chapter 1.

INTRODUCTION
CACHEXIA – THE SCALE OF THE PROBLEM

Cancer as a single entity is the second most common cause of death in the United Kingdom and indeed the Western World following ischaemic heart disease. It is estimated that there are 686 new cases of cancer diagnosed in the UK per 100,000 population per annum and the annual death rate for all malignant cancers is 513 per 100,000 per annum (CRC monograph, 1982). Cachexia to a varying degree is a common feature of malignant disease and leads to considerable morbidity and mortality (De Wys 1986, Warren, 1932).

The incidence of cachexia was studied, as part of the Eastern Oncology Co-operative Group investigations, in patients with solid malignancies (De Wys et al 1986). These investigations demonstrated that cancers could be stratified into those in which cachexia is a prominent feature and those in which cachexia is less common or less severe. A summary of these results is illustrated in Table 1.1.

Table 1.1
Frequency of weight loss in patients with different tumour types (Modified from De Wys 1986)

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Number of patients</th>
<th>Weight stable (%)</th>
<th>Weight-losing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>289</td>
<td>64</td>
<td>36</td>
</tr>
<tr>
<td>non-Hodgkin’s lymphoma</td>
<td>601</td>
<td>61</td>
<td>39</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>189</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>Colon</td>
<td>307</td>
<td>46</td>
<td>54</td>
</tr>
<tr>
<td>Prostate</td>
<td>78</td>
<td>44</td>
<td>56</td>
</tr>
<tr>
<td>Lung small cell</td>
<td>436</td>
<td>43</td>
<td>57</td>
</tr>
<tr>
<td>Lung non-small cell</td>
<td>590</td>
<td>39</td>
<td>61</td>
</tr>
<tr>
<td>Pancreas</td>
<td>111</td>
<td>17</td>
<td>83</td>
</tr>
<tr>
<td>Gastric</td>
<td>417</td>
<td>15</td>
<td>85</td>
</tr>
</tbody>
</table>
In general terms the cancers most frequently associated with accelerated weight loss are cancers of the gastrointestinal tract particularly gastric and pancreatic carcinoma followed by lung cancer and prostate cancer.

CLINICAL SIGNIFICANCE OF CACHEXIA IN THE CANCER HOST

Loss of lean tissue is associated with reduced physical capacity, depression, and in advanced disease is a significant cause of morbidity and mortality (Inagaki et al 1974). The physiological impairments and ultimately mortality associated with undernutrition are related to a loss of total body protein and this is reflected by the high incidence of hypostatic pneumonia as the terminal event in the starving patient (Moore 1980). Windsor and Hill (1988a) have shown that patients with more than 15% weight loss are likely to have clinically significant (i.e. >20%) loss of body protein. At this level of protein depletion they have shown that physiological function, including for example respiratory muscle function, is significantly impaired (Windsor & Hill, 1988a, Windsor & Hill, 1988b). In addition to impairment of respiratory muscle function, cachectic cancer patients have reduced muscle strength and decreased exercise tolerance (Windsor & Hill, 1988b). These factors may reduce the ability of the individual to maintain their independence and to perform activities of daily living. Less specific symptoms such as reduced cold tolerance, lethargy and fatigue are also common in the cancer-bearing host (Calman, 1982).

Nutritional depletion is associated with reduced resistance to infection with evidence of impaired cellular immunity (Bistain et al 1975). Furthermore, the presence of the tumour itself may also result in impaired T lymphocyte-mediated immune mechanisms with patients with gastrointestinal malignancy having reduced circulating IL-2 concentrations and reduced proliferation in response to T cell mitogens (Monson et al 1990). Profound protein depletion may also influence innate barrier defences such
as the gastrointestinal mucosal barrier and the respiratory epithelium which depend in part on rapid cell turnover to maintain their integrity.

Cachexia may influence both the ability of the patient to tolerate and to respond to treatment of the cancer. Marked depletion of lean tissue is associated with increased complications rates and mortality following surgery (Broden et al, 1984). Protein depletion in particular is associated with delayed wound healing (Haydock & Hill, 1986). All complications including infective, anastomotic leaks and thromboembolism are more common in the cachectic cancer host than in well nourished patients (Klidjian et al, 1980). In addition post-operative recovery is frequently prolonged and healing may be delayed (Kay et al, 1987). There is now considerable evidence that patients with cachexia are less tolerant of chemotherapy and radiotherapy and indeed these therapies may actually accelerate the progress of cachexia (Ollenschlager et al, 1992).

MECHANISMS OF CANCER CACHEXIA

ALTERED METABOLISM

Carbohydrate metabolism

Impaired glucose tolerance is a common feature of patients with malignancy Rossi-Fanelli et al, 1991). This intolerance is manifest by elevated serum glucose concentrations in the presence of normal or elevated serum insulin concentrations indicating an endogenous insulin resistance (Rothkopf, 1990). Furthermore patients with malignancy often have a suppressed response to treatment with exogenous insulin demonstrating a blunted suppression of blood glucose concentrations following insulin administration (Rothkopf, 1990). It has also been suggested that increased gluconeogenesis may contribute to impaired glucose tolerance and it is known that glucose can be synthesised by the liver and renal parenchyma from non-carbohydrate
precursors including lactate and alanine (Lawson et al, 1982). Some malignant cell lines have a high rate of anaerobic glucose oxidation to lactate and this lactate is processed by liver and kidney via the Cori cycle (Denis et al, 1985). This biochemical pathway is highly energy dependent utilising 6 moles of ATP per mole of glucose synthesised. This energy dependent substrate cycling has been implicated as an important drain on high energy phosphate bonds. Reicherd Waterhouse et al 1981 and others have demonstrated a 100% increase in glucose turnover in weight losing cancer patients while Cori cycle activity was 200% increased and lactate production was increased by 50%.

**Lipid metabolism**

Subcutaneous fat stores become markedly depleted in the cachectic cancer host. Loss of body lipid has been reported to occur both due to increased mobilisation of fat from fat stores (McAndrew, 1986) and from reduced lipogenesis (Jeevanandam et al, 1986). The primary source of this mobilisation is unknown although deranged insulin and glucagon activity has been suggested as a possible cause (Wesdorp et al, 1983, Lundholm et al, 1981). It has been shown that some of the alterations in the circulating lipid profile of cancer-bearing patients may be due to the effects of cytokines (McDonagh et al, 1992). TNF reduces low density lipoprotein (LDL) concentrations through lipid peroxidation (McDonagh et al, 1992) and LDL has been shown to stimulate TNF production by monocytes (Barath et al, 1990). In addition a number of reports have suggested the presence of tumour-derived lipid mobilising factors (Tisdale & Beck, 1991, Mulligan et al, 1992). In the MAC 16 colon cancer model a protein has been identified which is excreted in urine and which when purified and injected into non-tumour bearing animals leads to depletion of fat stores (McDevitt et al, 1995). This factor has since been isolated from the urine of cachectic patients with pancreatic cancer although it is a tumour-derived product, it is not yet known whether production of this factor can occur in normal tissues (Todorov et al, 1996).
Protein metabolism

Depletion of skeletal muscle mass is the most important consequence of cancer cachexia (Windsor & Hill, 1988a). Fearon and Preston (1990) have demonstrated that in lung cancer patients, skeletal muscle protein is the single largest source of protein depletion. Visceral and structural protein stores appear to be relatively well preserved in the weight losing cancer patient (Heymsfield, 1990). This different response of visceral and skeletal muscle protein suggests that some difference in the regulation of proteolysis in these tissues exists. One possible mechanism of such proteolysis may involve the ubiquitin-proteosome pathway (Mitch & Goldberg, 1996). The ubiquitin-proteosome pathway is thought to be the principal route by which skeletal muscle is broken down and it has been demonstrated that increased activation of this pathway occurs in some animal models of cancer cachexia (Baracos et al, 1995, Temparis et al, 1994).

Hypoalbuminaemia is another common observation in the weight losing cancer host (Calman, 1982). Controversy exists as to the exact reason for hypoalbuminaemia with early reports suggesting that both reduced hepatic albumin synthesis and increased fractional albumin catabolic rates were responsible (Brenner et al, 1990). More recent studies using tracer technology have demonstrated that the fractional albumin synthesis rate of patients with pancreatic cancer who are actively weight losing and are hypoalbuminaemic are no different or even increased compared with healthy age and sex matched controls (McMillan et al, 1996). Since 85% of total body albumin is localised in the extracellular compartment it has been proposed that hypoalbuminaemia may represent a shift from the vascular to the extracellular compartment and this effect is known to be responsible for the dramatic changes in albumin concentration which are sometimes associated with severe trauma and sepsis (Douglas & Shaw, 1989).
The cachexia syndrome is more complex than weight loss alone and includes a spectrum of symptoms including anorexia, early satiety, hypermetabolism, altered metabolic cycling, fluid retention and anaemia. The symptom patterns may vary widely between patients with different tumour types and between individual patients with the same tumour type.

*Body composition in cancer cachexia*

Analysis of body composition of cancer patients demonstrates that the principal component of weight loss is depletion of the skeletal muscle and fat compartments (Fearon & Preston, 1990) (Figure 1.1). In a study of body composition changes in lung cancer patients it was demonstrated that skeletal muscle protein was depleted in cancer patients by as much as 75% and subcutaneous fat stores by as much as 85% (Fearon & Preston, 1990). In the same study non-muscle protein stores remained the same as healthy controls thus structural and visceral protein is relatively well preserved (Figure 1.1). It is known that starvation causes the body to retain sodium and water, hence the chronically starved subject often shows much less weight loss than expected (Boulter et al 1973). In patients with cancer cachexia a state exists which is characterised by hypoalbuminaemia and a relative expansion of extracellular water space. Preston and Fearon in their study of lung cancer patients demonstrated that extracellular water accounted for 23% of body weight in healthy controls and 38% of body weight in cachectic cancer patients. This picture of wasting with hypoalbuminaemia may or may not be accompanied by clinical signs or oedema. Despite relative expansion of the extracellular fluid compartment, clinically available signs of fluid retention such as oedema or ascites are frequently not apparent until a very late stage in the natural history of cancers. The fact that total body water and extracellular water are relatively
well preserved in cachectic cancer patients may lead to underestimation of the severity of their nutritional status.

Figure 1.1
Differences in body composition between healthy controls and patients with lung cancer (Adapted from Fearon, 1992)

The concept of energy balance

The mechanisms of weight loss in patients with pancreatic cancer are complex but ultimately lead to a negative energy and nitrogen balance. Body weight is at the most basic level the result of a balance between energy intake and energy expenditure. Energy intake can be considered to be a single compartment model since only dietary intake and absorption of food are its determinants. Energy expenditure can be considered to have two principal compartments. Firstly, there is a basic energy requirement needed to maintain life. The basal metabolic rate or resting energy
Expenditure (REE), is the energy expenditure of a fasted subject at rest and in a thermoneutral environment. It composed of the energy required for maintenance of circulation, respiration, cerebral activity, metabolism and maintenance of core temperature. Secondly, there is a voluntary component of energy expenditure which is determined by the level of activity of the individual. Voluntary energy expenditure (VEE) is accounted for principally by muscular work. The proportion of total energy expenditure (TEE) accounted for by REE and VEE varies depending on the activity of the individual. In a sedentary person REE would account for as much as 75% of TEE (Figure 1.2), whereas in an active individual TEE will be considerably higher and since this results from increased VEE the fraction of TEE accounted for by REE may be as little as 30%. Approximately 10% of TEE is accounted for by the thermogenic response to feeding which represents the expenditure in the metabolic processing of food (Westrate et al, 1989).

Figure 1.2
Proportions of total energy expenditure accounted for by diet induced thermogenesis, resting and voluntary energy expenditures in elderly sedentary patients.
Since body weight may be crudely considered as the result of energy balance, weight loss may result from either a reduced energy intake or an increased energy expenditure or a combination of the two (Figure 1.3). Assuming that nutritional deletion can be explained predominantly in terms of energy balance, rate of depletion will be dependent on the magnitude of the total energy deficit.

Figure 1.3

Factors influencing energy balance in patients with cancer

An early study of metabolic rate in patients with gastrointestinal cancer failed to demonstrate any significant difference compared with controls with benign inflammatory gastrointestinal disease (Burke et al, 1980). On the basis of this data the authors of this report concluded that weight loss in gastrointestinal cancer was probably due to reduced energy intake. Other studies demonstrated however, a significant association between elevation of metabolic rate or resting energy expenditure and magnitude of weight loss (Bozetti et al, 1980). Koea and Shaw (1991) attempted to calculate tumour burden in patients with gastrointestinal cancer and were able to demonstrate a significant correlation between tumour bulk and REE. A study of energy balance in patients with lung cancer demonstrated that these patients had significantly elevated REE and a relatively low energy intake compared with a group of
predominantly colon cancer patients who had a relatively normal REE and low energy intake (Fredrix et al, 1990). It is now generally accepted that elevation of REE is a common feature of certain tumour types and may contribute to energy deficit leading to weight loss.

FACTORS LEADING TO REDUCED NUTRITIONAL INTAKE

Anorexia and early satiety may be caused by a variety of clinical factors including gastric outlet obstruction, osychogenic nausea and vomiting, oral ulceration, altered taste sensation, conditioned taste aversions, depression and anxiety (Calman, 1982). It is evident, however, that in a large proportion of cancer patients, anorexia occurs in the absence of obvious physical or psychological disturbance. The hypothalamic appetite and satiety centres are regulated by a variety of humoral mediators. Changes in plasma free fatty acids glucose and amino acid concentrations are known to influence appetite regulation. In animal models 5HT has been associated with anorexia (Stallone et al, 1989) and it has been proposed that increased binding of tryptophan to albumin might increase the cerebral precursor pool for 5HT (Laviano et al, 1996a; Laviano et al, 1996b). There is also increasing evidence that cytokines may influence central appetite regulation (Tracey et al, 1988; Gelin et al, 1989; Sherry et al, 1989). Animal studies have demonstrated that infusion of pro-inflammatory cytokines such as tumour necrosis factor alpha is associated with anorexia and that this reduction of nutrient intake can be reversed by the administration of specific antibodies to TNF (Sherry et al, 1989; Smith & Kluger, 1993). Whether cytokines exert a central effect on appetite regulation or whether they act through an intermediary pathway is unclear (Laviano et al, 1996b).

Recent research has revealed a homeostatic pathway responsible for regulation of fat mass (Collins et al, 1996). A polypeptide hormone called leptin has been
identified which is produced by adipocytes in direct proportion to fat mass (Ronnemaa et al, 1997). This hormone has been shown to be capable of crossing the blood brain barrier and to bind to receptors on the arcuate nucleus (Schwartz et al, 1996). The presence of high leptin concentrations stimulates a neuroendocrine pathway between the arcuate and paraventricular nuclei which results in release of the catabolic hormone corticotrophin releasing hormone which results in reduced food intake, reduction in body weight, increased thermogenesis and reduced lipogenesis (Satoh et al, 1997). By contrast in the fasted state reduced leptin concentrations results in production of the anabolic hormone neuropeptide Y which results in increased food intake, weight gain, reduced thermogenesis and increased lipogenesis in an attempt to restore body weight (Wang et al, 1996; Kennedy et al, 1997). This research has been conducted principally in rodents however leptin has been identified in man and it is possible that a similar regulatory pathway may influence body weight in man. It has been suggested that the leptin-hypothalamic pathway might be influenced by cytokines or counter-regulatory hormones such that the system were less responsive to reduced leptin concentration or alternatively biased toward catabolic hormone production (Grunfeld et al, 1996; Zakrzewska et al, 1997).

In cancer patients oral ingestion of food does not guarantee that the protein and energy content of the food will reach the target organs. Delayed gastric emptying, vomiting secondary to chemotherapy or radiotherapy, pancreatic exocrine insufficiency and fat malabsorption can contribute to a decreased energy intake.

THE CYTOKINE/ACUTE PHASE PROTEIN RESPONSE IN CANCER CACHEXIA

The mechanisms whereby the metabolism of the cancer host becomes altered have been studied intensively over the past 70 years. However, recent impetus has been given to the field of inter-cellular messengers known as cytokines. First described in
relation to regulation of the immune system, cytokines are now recognised to be produced both by tumour cells and by host cells in response to the tumour. As mentioned previously, pro-inflammatory cytokines such as interleukin-1, interleukin-6 and tumour necrosis factor can act centrally to alter appetite regulation. It is also of interest that such cytokines appear to regulate key events in the host response to injury. One such event is the hepatic acute phase protein response (APPR). The liver is the centre for acute phase protein metabolism. The acute phase proteins are named after the observation that their serum titre changes in response to certain physiological events (Kushner, 1986). Stress, trauma, burns and sepsis all produce alterations in liver protein metabolism particularly affecting the hepatic acute phase proteins (Heinrich, 1990). The acute phase proteins are divided into positive (serum concentration increases in response to stress) and negative (serum concentration decreases in response to stress) Table 1.2. The principal positive acute phase proteins are, C-reactive protein, α1-antichymotrypsin, α1-acid glycoprotein (or somucoid), serum amyloid A, caeruloplasmin and haptoglobin. The principal negative acute phase proteins are albumin, prealbumin and transferrin.

Table 1.2
Changes in production rates of acute phase proteins by human hepatocytes in response to IL-6 (Modified from Heinrich et al, 1990)

<table>
<thead>
<tr>
<th>Increase</th>
<th>magnitude (fold)</th>
<th>Decrease</th>
<th>magnitude (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein</td>
<td>10-100</td>
<td>Albumin</td>
<td>&lt;2</td>
</tr>
<tr>
<td>serum amyloid A</td>
<td>10-100</td>
<td>Prealbumin</td>
<td>&lt;2</td>
</tr>
<tr>
<td>α1-antichymotrypsin</td>
<td>2-10</td>
<td>Transferrin</td>
<td>&lt;2</td>
</tr>
<tr>
<td>α1-acid glycoprotein</td>
<td>2-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fibrinogen</td>
<td>2-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>caeruloplasmin</td>
<td>&lt;2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>haptoglobin</td>
<td>&lt;2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Overall the APPR represents a reprioritisation of protein synthesis away from peripheral tissues (e.g. skeletal muscle) toward the production of export proteins by the liver. These proteins function to facilitate tissue healing and repair e.g. opsonisation of bacteria, anti-protease activity, transport of metal ions and as essential components of the clotting cascade.

In acute illness, the APPR forms part of the metabolic response to stress and is generally thought to be of benefit provided that the response is not prolonged or excessive. In chronic illness there is increasing evidence that prolonged activation of the APPR may be detrimental. The “auto-cannibalism” of skeletal muscle which occurs in severe acute injury and in chronic illness has been explained in teleological terms as providing a resource of amino acids for gluconeogenesis (Douglas & Shaw, 1989). In patients with rheumatoid arthritis, cachexia has been associated with the presence of persistent elevation of the APPR (Roubenoff et al, 1994). Similar associations have been described in relation to cardiac cachexia and in weight losing patients with chronic obstructive pulmonary disease. It has previously been demonstrated that weight-losing patients with pancreatic cancer frequently have an elevated hepatic acute phase protein response compared with healthy control subjects. (Basso et al, 1988; Falconer et al, 1994). This phenomenon has also been observed in patients with other cancers including small cell lung cancer, colorectal cancer, ovarian and renal cell carcinoma. At the time of diagnosis approximately one half of patients with unresectable pancreatic cancer have an APPR (Basso et al, 1988). The APPR increases both in incidence and intensity with disease progression such that at the time of death 78% of patients with pancreatic cancer will have an elevated APPR (Falconer et al, 1995). The presence of an APPR at the time of diagnosis has also demonstrated to be the single strongest independent predictor of poor prognosis exceeding the prognostic value of all conventional markers of disease such as anatomical tumour stage and age (Falconer et al, 1995). In the latter study patients who had an acute phase response had a median survival of 66 days whereas those who did not have an elevated APPR had a median
survival of 222 days. Thus it would seem that in that study the biological consequence of the host-tumour interaction was of greater prognostic significance than tumour spread per se.

*Isolated human hepatocyte cultures*

The isolation and primary culture of human hepatocytes provides one means of studying the acute phase protein response. Techniques for isolation and purification of hepatocytes have been described (Berry et al, 1991; O’Riordain et al, 1995) which permit cultures to be established with maintenance of protein synthetic function for at least 4 days. Heinrich et al (1990) have characterised the acute phase protein response using an in vitro human hepatocyte system and have confirmed that such cultures produce the full range of acute phase proteins which are produced by the liver in vivo. This offers a clear advantage over studying human hepatoma cell lines such as Hep G2, Hep 3B and HUH7 since none of these cell lines produce all of the human acute phase proteins (Table 1.3).

Table 1.3
Variations in patterns of production of a selection of acute phase proteins by adult human hepatocytes and two hepatoma cell lines in response to the addition of IL-6. ++++, ++, + = increase production; - = decreased production and 0 = not produced; nd = not determined *(Adapted from Heinrich et al., 1990)*

<table>
<thead>
<tr>
<th></th>
<th>Human hepatocytes</th>
<th>Hep G2 cells</th>
<th>Hep 3B cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein</td>
<td>+++</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>serum amyloid A</td>
<td>+++</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>α1-antichymotrypsin</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>α1-acid glycoprotein</td>
<td>++</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>fibrinogen</td>
<td>++</td>
<td>+++</td>
<td>nd</td>
</tr>
<tr>
<td>caeruloplasmin</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>haptoglobin</td>
<td>++</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Transferrin</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>
There is evidence that IL-1 and TNF induce the upregulation of IL-6 production (Shalaby et al, 1989; Shimizu et al, 1990) and that IL-6 is the principal cytokine involved in delivering the final signal which results in alteration of hepatocyte acute phase protein synthesis (Heinrich et al, 1990; Baumann et al, 1992). Furthermore IL-6-induced transcriptional activation (Morrone et al, 1988) of a set of human hepatocyte acute phase protein genes and nuclear transcription factors which interact with the C-reactive protein promoter site (Majello et al, 1990) have been described. This IL-6 induced transcriptional activation appears to operate at the pre-transcription level (Castell et al, 1990). O'Riordain et al (1995) have demonstrated that human acute phase protein production can be influenced by the action of counter-regulatory hormones in vitro. This study demonstrated that insulin suppressed IL-6-induced acute phase protein production and that adrenaline glucagon and cortisol augmented acute phase protein production. Similar results had been previously obtained using insulin in a study of acute phase protein production by a hepatoma cell line (Thompson et al, 1991).

The acute phase protein response and skeletal muscle protein

The precise effects of the acute phase response on nutritional status are unclear. It has been demonstrated that a significant correlation exists between the presence of an elevated APPR and increased resting energy expenditure in patients with pancreatic cancer (Falconer et al, 1994). Furthermore, it has been suggested that acute phase protein production may accelerate skeletal muscle protein depletion because acute phase proteins contain a different mixture of amino acids from that observed in skeletal muscle, the latter being the principal labile amino acid reserve in the body (Reeds et al, 1994). Acute phase proteins are rich in aromatic amino acids such as tryptophan and phenylalanine while skeletal muscle contains low concentrations of these amino acids. Thus competition for the limited amino acid pool consequent on the reprioritisation of body protein metabolism may account for a disproportionate breakdown of skeletal muscle to fuel the acute phase protein response (Reeds et al, 1994; Preston et al, 1996).
Production of pro-inflammatory cytokines by peripheral blood mononuclear cells.

Augmented production of hepatocyte-stimulating cytokines has been described by peripheral blood mononuclear cells (PBMC) of weight-losing patients with pancreatic cancer (Falconer et al, 1994). In that study, both spontaneous and endotoxin-stimulated cytokine production was greater in the PBMC derived from pancreatic cancer patients with an acute phase response compared with those who did not have such a response or compared with healthy controls. Despite augmented production of pro-inflammatory cytokines by PBMC of cancer patients with an elevated APPR, serum concentrations of the same cytokines did not correlate with an APPR. This has lead to the consideration that concentrations of cytokines in tissues may be of greater importance than circulating concentrations. The mechanism of activation of these peripheral blood mononuclear cells is uncertain but may represent either a non-specific immune response to the tumour itself or priming as a consequence of chronic low dose exposure to endotoxin.

T-lymphocyte derived cytokines such as Interleukin 4 and interleukin 10 have been shown to down regulate production of IL-6 and TNF by peripheral blood mononuclear cells (Essner et al 1994, Lee et al 1990, Fiorentino et al 1991) and to alter the phenotype of such cells (teVelde 1988). It has been proposed that these lymphokines may form a homeostatic mechanism regulating pro-inflammatory cytokine production by monocytes. It has been demonstrated that T cell function is impaired in patients with gastrointestinal malignancy (Monson et al 1990) and multiple organ failure. One such manifestation of impaired T-cell function is the decreased production of IL-2. IL-2 is the principal stimulator of IL-4 and IL-10 production by human lymphocytes and as such can be considered to be the principal regulator of T-lymphocyte function. Since IL-4 and IL-10 suppress pro-inflammatory cytokine production, failure of T-cell mediated regulation of pro-inflammatory cytokine release might therefore contribute to abnormal control of the acute phase response in disease. It
is possible therefore that enhancing the IL-4 mediated suppression of pro-inflammatory cytokine production might be of potential benefit in cancer cachexia by attenuating the APPR (Figure 1.4).

Figure 1.4
Diagram illustrating the inter-relationship between regulatory and pro-inflammatory cytokines in relation to the aetiology of the acute phase protein response

Recently it has become evident that a variety of tumour cells themselves may be capable of producing cytokines with the potential to elicit an acute phase response. The production of pro-inflammatory cytokines has been described by a number of human tumour cell lines. IL-6 production has been identified in human gastric (Baba et al, 1995), myeloma (Westendorf et al, 1996), oral squamous cell carcinoma (Matsuo et al, 1994), acoustic neuroma (Adams et al, 1994), astrocytoma (Kasahara et al, 1990) and renal cell carcinoma (Muraki & Nakazano, 1992) cell lines. IL-8 production has
similarly been described in a number of different human tumour cell lines including gastric (Yasumoto et al, 1992), an astrocytoma (Kasahari et al, 1991) and a thyroid carcinoma cell line (Yoshida et al, 1992). In the case of cell lines producing IL-8, production of this cytokine was dependent on stimulation with TNF or IL-1 whereas all of the cell lines producing IL-6 do so constitutively. TNF production has not been described by any human tumour cell line and since TNF is thought to have tumoricidal properties this is perhaps not surprising. Whether tumour cell cytokine production occurs in vivo in man is uncertain. Identification of such cytokine production is technically difficult particularly because the infiltrate of immune and inflammatory cells associated with tumours makes identification of the source of any cytokines difficult. In addition techniques such as immunohistochemistry which have been used to identify tissue cytokine production are not sufficiently accurate. IL-6 production has been identified in vivo in an animal tumour, the murine colon 26 adenocarcinoma (Strassman et al, 1993). In this model IL-1 stimulates IL-6 production and by treating animals with IL-1 receptor antagonist, partial inhibition of tumour cell IL-6 release can be achieved (Strassman et al, 1993).

Whatever the mechanism of enhanced pro-inflammatory cytokine production in cancer, the implication of such cytokines in both anorexia and the metabolic changes contributing to weight loss suggest that either inhibition of cytokine production or of end-organ effects may be a key method of intervention in the anorexia-cachexia syndrome and such an approach will be discussed in detail later.

DIRECT CACHECTIC FACTORS

Recent evidence has suggested that factors may be elaborated by tumours which lead to direct lipolysis and proteolysis of fat and skeletal muscle protein stores (Mulligan et al, 1992; Smith & Tisdale, 1993). In the Mac 16 murine colon adenocarcinoma model weight loss is observed in the presence of a normal nutritional
intake and in the absence of pro-inflammatory cytokines (Mulligan et al, 1992). A factor has been identified in the urine of these mice which when purified and injected into non-tumour bearing animals results in weight loss with evidence for direct proteolysis and to a lesser extent lipolysis (McDevitt et al, 1995, Todorov et al 1996a). This factor has subsequently been identified in the urine of weight losing patients with pancreatic cancer and other malignancies including lung cancer (Todorov et al, 1996b). Purification of this moiety has shown it to be a 24kD protein with heavy glycosylation (Todorov et al, 1997). Injection of the factor derived from the urine of human patients with malignancy results in weight loss in non-tumour bearing animals in a similar fashion to the mouse protein. The precise mechanism of action of this protein is as yet unknown although preliminary evidence suggests that it induces proteolysis via binding to a specific receptor expressed by skeletal muscle.

CARCINOMA OF THE PANCREAS AS A MODEL OF CANCER CACHEXIA

Pancreatic cancer is now the fifth most common cause of cancer death in the Western World (Williamson, 1988). In the United Kingdom the average incidence of pancreatic cancer is 8 per 100,000 and there are approximately 6000 deaths per annum (CRC Monograph, 1982). Adenocarcinoma of the pancreas is a disease of old age with a median age of presentation of 70 years. The majority of pancreatic cancers occur in the head of the gland (82%) with 16% occurring in the body and 2% in the tail (Becker & Stommer, 1993). Most tumours arise from the ductal system (80%) and the majority of lesions are adenocarcinomas (>80%). Adenocarcinomas of the pancreas are frequently associated with an intense desmoplastic response and associated surrounding zone of pancreatitis. This tissue inflammation may account for two thirds of the bulk of the tumour and makes accurate staging of the neoplastic tissue difficult.

The prognosis for patients with adenocarcinoma of the pancreas is poor with median survival of between 3 and 6 months following diagnosis and few patients
surviving more than one year (Williamson, 1988; Cancer Of The Pancreas Task Force, 1981). Therapeutic options for pancreatic cancer patients are extremely limited. Surgical resection of the tumour offers the best survival advantage with 1 year survival rates of 35 and 40% reported by specialist units. Unfortunately, due to local invasion or metastatic disease, surgical resection is not possible in 90% of patients (Carter, 1989). Radiotherapy has been used in patients with pancreatic cancer both as primary therapy (Zerbi et al, 1994) and in conjunction with resectional surgery (GITSG, 1987) and or chemotherapy (GITSG, 1981; Jeekel & Treurniet-Dornier, 1991). Adenocarcinomas in general are not radiosensitive and pancreatic cancer rarely responds to radiotherapy. A variety of chemotherapeutic strategies have been utilised in pancreatic cancer. Two studies have demonstrated a survival benefit from combination chemotherapy of cyclophosphamide, methotrexate and 5 fluorouracil (Mallinson et al, 1980; Palmer et al, 1994). The benefit in both studies was small in terms of overall survival but there was a significant interim survival advantage at 3 months and 6 months compared with the non-treatment group. Combination chemotherapy has significant disadvantages in terms of the morbidity associated with treatment and in patients with a limited life expectancy this factor is very important. The incidence of complications and side effects related to treatment was considerable in both of the trials which showed a benefit. The limitations of chemotherapy and radiotherapy are perhaps best highlighted by the fact that none of these treatments has become accepted clinical practice.

TRIALS OF INTERVENTION IN CANCER CACHEXIA

Supplementation of intake

The most obvious means to tackle the problem of cachexia would be to provide additional calories and protein to ensure that requirements are met or exceeded. A number of studies have attempted to improve nutritional status in such patients by the
provision of additional protein and energy in the form of nutritional supplements. Studies can be divided into those which have utilised the patients own gastrointestinal tract, enteral nutrition, via naso gastric or nasojejunal feeding and those of parenteral nutrition using central or peripheral venous feeding. Under normal circumstances hyperalimentation would be expected to result in universal weight gain or weight stabilisation.

Although early uncontrolled trials of TPN in cancer patients suggested clinical benefit from this approach these results have not been substantiated by controlled trials. Early controlled trials demonstrated no nutritional benefit from enteral or parenteral nutritional support however quality of life was reported to have been improved. Since the early studies over 20 randomised trials including over 800 patients have been conducted in which duration of TPN administration ranged from 7 to 70 days. The results demonstrated that TPN did not improve outcome of surgery, chemotherapy or radiotherapy in patients with cancer (Brennan et al, 1994; Ng & Lowry, 1991; Heys et al, 1992). Furthermore, TPN did not increase fat free mass or total body potassium and at best only increased body weight or reduced rate of weight loss. Studies of hyperalimentation using enteral or parenteral supplementation have thus been disappointing with only a proportion of patients gaining weight (Cohn et al, 1982) and the principal component of such weight gain being water and fat (Bosetti, 1992). Possible explanations for this apparent block to accretion of lean tissue include abnormalities of substrate utilisation such as increased energy -dependent substrate cycling (c.f. gluconeogenesis) or altered hormonal/cytokine regulation of protein synthesis in lean tissue (Nixon et al, 1981).
SPECIFIC ANTI-CACHECTIC STRATEGIES

The search for a therapeutic strategy which is effective in attenuating or reversing cachexia associated with cancer has led to the trial of a number of different agents with a specific mode of action.

Corticosteroids.

Corticosteroids such as prednisolone, methylprednisolone and dexamethasone have been widely used in patients with advanced malignancy (Ettinger & Portenoy, 1988). The rationale behind their use is based on three principal observations. Firstly the observed improvement in sense of well-being which many patients receiving corticosteroids experience. Secondly subjective improvement in appetite and thirdly their potential to attenuate tumour-associated inflammatory processes through their anti-inflammatory actions. Trials have confirmed that appetite scores do improve when patients are treated with corticosteroids however this does not translate into improvements in body composition or weight gain (Willox et al, 1984). Indeed corticosteroids have catabolic activity towards skeletal muscle protein and may potentially accelerate lean tissue wasting (Kayali et al, 1987). Furthermore the commonly used corticosteroids also have mild mineralocorticoid activity resulting in fluid retention making assessment of weight change difficult in the absence of adequate body composition analysis.

Hydrazine sulphate.

Hydrazine sulphate was initially used in patients with cancer because it was thought to have anti-neoplastic activity (Gold et al, 1968), this hypothesis was later disproved by a number of clinical trials (Gershanovich et al 1981). It was thought to have some potential benefits in cachexia for two reasons. Firstly, since hydrazine sulphate is an inhibitor of phosphoenol pyruvate kinase; the enzyme responsible for converting glucose to lactate; it was thought that it might have a glucose sparing effect.
Secondly hydrazine sulphate has a direct antagonistic effect to some of the actions of TNF which is known to have a role in the mediation of cachexia particularly in animal models (Hughes et al, 1989). Early trials suggested that use of hydrazine sulphate was associated with maintenance of body weight and reduction in whole body protein breakdown, however, more recent studies have failed to confirm these observations and have shown no clinical benefit from treatment with this drug (Chlebowski et al 1987).

**Tetrahydrocannabinol (THC)**

Tetrahydrocannabinol and other cannabinoids, which are extracted from the cannabis plant, have been used for their antiemetic effects in patients with advanced malignancy. Cannabinoids have also been considered to have some anabolic properties and might therefore have a role in cancer cachexia however there are no controlled studies which can validate this claim (Bruera, 1992; Nelson et al, 1994).

**Insulin infusions**.

Cancer cachexia is associated with increased resistance of peripheral tissues to insulin and persistent hyperglycaemia. In animal models of cancer cachexia insulin infusion appears to be of benefit and it has been proposed that it might also be of benefit in human malignancy (Moley et al, 1988). No controlled trials of insulin have been undertaken however the small studies which have used this approach observed normalisation of biochemistry without any substantial effect on the progress of cachexia (Cerosimo et al 1991; Byerley et al, 1991).

**Cyproheptadine**.

Cyproheptadine is a serotonin and histamine antagonist which has some appetite-stimulating properties. Since elevated serotonin has been associated with anorexia, it was thought that a serotonin antagonist might have potential in cancer cachexia. A controlled trial however demonstrated that although appetite was improved no benefit was observed in relation to weight loss (Kardinal et al, 1990).
**Pentoxyfylline**

Pentoxyfylline has anti-TNF properties and appears to act via suppression of TNF RNA synthesis rather than as a direct antagonist. Small early studies using pentoxyfylline in patients with malignancy reported improvement in appetite and weight gain (Dezube et al, 1993). A large randomised controlled trial has subsequently failed to identify benefit in terms of weight gain or improvement in body composition (Goldberg et al, 1995). This study did not however confirm that there was an effect on TNF at the dose range studied.

**Clenbuterol**

Clenbuterol is an adrenergic beta2 receptor agonist which is thought to inhibit catabolic and cachectic processes. It has been shown to improve muscle function in patients undergoing orthopaedic surgery (Mallin et al, 1993) and to have anti-catabolic effects in animal models (Carbo et al, 1997) but has not been evaluated in human cancer cachexia.

**Medroxyprogesterone acetate**

MPA has been used as hormonal therapy in the treatment of patients with breast cancer (Nemotol et al, 1986). Such treatment has been associated with weight gain and stimulation of appetite. In a controlled trial of MPA in hormone-insensitive cancer cachexia, marked improvement of appetite was recorded compared with pre-treatment however, no improvement was observed with respect to weight, performance status or quality of life (Downer et al, 1993; Slevin et al, 1988).

**Nandrolone decanoate**

Nandrolone decanoate is an anabolic steroid which has been shown to promote skeletal muscle hypertrophy and as a result has been abused in sport as a performance enhancing agent. Because of its anabolic properties it has been trialled in patients with
small cell lung cancer, however, this study did not demonstrate a treatment benefit (Chlebowski et al, 1986). In animal models of cancer cachexia nandrolone produced weight gain due to fluid retention but did not increase lean tissue mass or survival duration (Lyden et al, 1995).

Megestrol acetate.

Is a progestational agent used for the treatment of hormone dependent cancers. In a similar way to medroxyprogesterone acetate its use in patients with breast cancer was associated with stimulation of appetite and weight gain (Aisner et al, 1987). Unlike medroxyprogesterone acetate, its use in gastrointestinal malignancy has also been associated with improvement in appetite and some improvement in weight gain (Schmoll et al, 1991, Loprinzi et al 1993, Tchemedjian et al 1992, Feliu et al 1991, McMillan et al, 1994). Megestrol acetate is discussed in more detail in Chapter 8.

A wide range of different drugs have been used to treat cancer cachexia. Despite small improvements in one or more of the nutritional indices studied no drug has produced consistent attenuation of cachexia or has become an accepted agent for clinical treatment of weight-loss in cancer.

Ibuprofen

Ibuprofen is a non-steroidal anti-inflammatory drug which acts by inhibiting the enzyme cyclo-oxygenase which is a central point in the metabolic pathway of eicosanoids and prostaglandins. It is known to inhibit some of the end organ effects of the proinflammatory cytokines IL-6, IL-1 and TNF (Dinarello & Wolff, 1982, Durum et al, 1985). In particular, ibuprofen has been shown to reduce body temperature and the metabolic rate of patients with burn injury (Wallace et al., 1992) and to reduce the level of the acute phase response in some patients with rheumatoid arthritis (Cash et al, 1990). Furthermore, in sepsis, patients pre-treated with ibuprofen show an attenuated TNF response to endotoxin challenge (Spinas et al, 1991; Matrich et al, 1991). Despite
evidence that ibuprofen can inhibit the end organ effects of cytokines, other studies have demonstrated paradoxical elevation of pro-inflammatory cytokine production by isolated peripheral blood mononuclear cells following treatment with ibuprofen (Kunkel et al, 1986; West et al, 1993). In weight losing patients with cancer administration of ibuprofen was found to reduce acute phase protein production and whole body protein kinetics (Preston et al, 1995). Moreover long term administration of ibuprofen to patients with cancer was shown to reduce serum concentrations of IL-6, cortisol and C-reactive protein (McMillan et al, 1995). The effects of ibuprofen on weight loss have not however been studied.

The polyunsaturated fatty acids

It has been suggested that certain polyunsaturated fatty acids (PUFAs) may have a therapeutic role as anti-inflammatory and anti-neoplastic agents. Eicosapentaenoic acid (EPA) has a 20 carbon atom chain containing 5 double bonds (C20:5 n3) and occurs naturally as a major component of fish oil. Gammalinolenic acid (GLA) has an 18 carbon atom chain containing 3 double bonds (C18:3 n6) and is derived predominantly from seed oils. EPA and GLA and their metabolic precursors linoleic acid (C18:2 n6) and alphalinolenic acid (C18:3 n3) are essential fatty acids and are thought to have an important role in regulating the fluidity of cell membranes (Hwang, 1989) and act as substrates for prostaglandin synthesis (Fisher et al, 1985). The administration of such fatty acids has been associated with alterations in various aspects of the immune and inflammatory response (Hwang, 1989). The mechanisms by which fatty acids exert their immunomodulatory effects are unclear but appear to be diverse.

Administration of PUFAs to healthy volunteers has been shown to downregulate monocyte derived-interleukin-1 (IL-1) and tumour necrosis factor (TNF) production (Endres et al, 1989) and to inhibit neutrophil and monocyte degranulation, phagocytosis and enzyme release (Virella et al, 1989). Further studies have
demonstrated that dietary fish oil inhibits tachycardia and attenuates the maximal increases in temperature and metabolic rate following administration of typhoid vaccine (Cooper et al, 1993). Furthermore IL-1 and IL-6 production following typhoid vaccine administration is suppressed in volunteers treated with fish oil (Cooper et al, 1993). The antipyretic effects of fatty acids cannot however be explained simply by a reduction in IL-1 production, since animals fed with a PUFA-rich diet have an attenuated febrile response to the infusion of IL-1 (Pomposelli et al, 1989). This implies that PUFAs may not only act by modulating monocyte pro-inflammatory cytokine release, but also by changing end-organ responsiveness to cytokine stimulation.

In addition to the anti-inflammatory effects of polyunsaturated fatty acids on cytokine-mediated pathways, there is clinical evidence of immunomodulatory activity resulting from the administration of these drugs, the mechanism of which is uncertain. Cerra et al (1990) have demonstrated that enteral supplementation containing EPA and other PUFA’s is associated with improvement of immune function and reduction in incidence of infective complications in patients in an intensive care unit setting. It has also been demonstrated that administration of fish oil to patients with rheumatoid arthritis is associated with reduction in joint pain scores and improvement of joint mobility. Further more fish-oil-supplemented enteral nutrition was associated with a 50% reduction in gastrointestinal complications and improvement of renal and hepatic function in post surgical cancer patients (Kenler et al; 1996).

It has been suggested that one of the major effects of PUFAs in vivo is to modify the synthesis of eicosanoids and prostanoids. Both EPA and GLA have been shown to alter prostaglandin synthesis by competing with arachidonic acid for cyclooxygenase and lipoxygenase enzymes. Arachidonic acid is normally converted into the 2 series prostaglandins, the competitive effect of EPA and GLA results in diversion of prostaglandin synthesis away from the 2 series toward the 1 series and 3 series respectively (Fischer et al, 1985). These alterations in prostaglandin synthesis
have been considered to be associated with an attenuation of the inflammatory response (Hwang, 1989). It is unclear whether series 1 and series 3 prostaglandins have an anti-inflammatory effect on human hepatocyte acute phase protein production directly (Wigmore et al, 1994). Essential fatty acid rich diets are associated with a diminution of PGE2 production and the potential benefits of this action are unclear (Ferreti & Flanagan, 1990). PGE2 has been demonstrated to down-regulate monocyte TNF (Kunkel et al, 1988; Spengler et al 1989) and IL-1 production (Kunkel et al; 1986). Furthermore it has been demonstrated that PGE2 appears to have a protective action in the human liver by downregulating Kupffer cell pro-inflammatory cytokine production in an autocrine fashion (Roland et al, 1994; West et al, 1993; Goss et al, 1992) and would thus appear to be a naturally occurring regulator of the inflammatory response. Therefore it would seem unlikely that reducing PGE2 production would have a beneficial anti-inflammatory effect.

The polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and gamma-linolenic acid (GLA) inhibit growth of a variety of human carcinoma cells in vitro (Begin et al, 1985; Begin et al, 1986; Begin et al, 1988; Dippenaar et al, 1982; Karmali et al, 1984; Karmali et al, 1987; Wica et al, 1987) including human pancreatic cancer cell lines (Falconer et al, 1994). Diets supplemented with PUFA inhibit the growth of solid tumours in a variety of tumour-bearing mouse models. In addition, EPA is capable of attenuating the weight loss in the MAC16 murine model of cachexia by a mechanism which appears to be distinct from the anti-tumour effect (Hudson & Tisdale 1994; Tisdale et al, 1994; Tisdale, 1996).

AIMS OF THIS THESIS

This thesis investigates the mechanisms underlying cancer cachexia using pancreatic cancer as a model. In order to define the model a longitudinal study was
undertaken to evaluate the progression of weight loss and nutritional depletion in a group of untreated patients with pancreatic cancer. Weight loss in general terms may be considered to arise from a negative energy balance, itself a consequence of either reduced energy intake or increased energy expenditure. Recent evidence has suggested a relationship between cytokine mediated events, particularly the acute phase protein response and altered energy balance in patients with cancer cachexia. The components of energy deficit contributing to weight loss in patients with pancreatic cancer cachexia and their relationship to the presence of an acute phase response were therefore investigated.

A series of in vitro studies were then undertaken to attempt to identify sources of augmented pro-inflammatory cytokine production in cancer cachexia and their effect on the APPR. The production of pro-inflammatory cytokines by human pancreatic tumour cell lines and peripheral blood mononuclear cells isolated from patients with pancreatic cancer and their effect on acute phase protein production in an in vitro model using isolated human hepatocytes is investigated. Reports have suggested that regulatory cytokines such as IL-4 and IL-10 can down-regulate the production of pro-inflammatory cytokines such as IL-6 and TNF. If pro-inflammatory cytokines are indeed detrimental in cancer cachexia, therapeutic down-regulation of their production by immunomodulation might be an effective therapeutic strategy. The effects of IL-4 and IL-2 on pro-inflammatory cytokine production and the acute phase protein response was therefore examined. An attempt was also made to establish whether increased concentrations were present in the sera of patients with pancreatic cancer and what if any was the relationship between such cytokines and the APPR.

The effects of a number of novel therapeutic strategies directed to attempt to attenuate cachexia is described. In the first study ibuprofen, a drug which is known to reduced pro-inflammatory cytokine production was used to attempt to down regulate the acute phase response to establish whether this would result in a reduction in resting energy expenditure. A further study was then taken combining ibuprofen with the appetite stimulant megestrol acetate to establish whether longer term administration of
ibuprofen resulted in weight gain compared with placebo. Polyunsaturated fatty acids have also been shown to reduce the production of pro-inflammatory cytokines by immune cells. A study was undertaken using a crude preparation of fish oil containing 18% eicosapentaenoic acid (EPA) to examine the effects of this drug on nutritional indices in patients with advanced pancreatic cancer. A further study was undertaken using highly purified EPA in cancer cachexia at a higher effective dose than given in the study of fish oil. This study sought to establish whether EPA was an effective anti-cachectic agent, whether any dose response effect was evident and to identify its potential mechanism of action using parallel in vitro studies.
Chapter 2.

FREQUENTLY QUOTED METHODS
ISOLATION OF HUMAN HEPATOCYTES

MATERIALS

Perfusion apparatus-Equipment List

Buschner funnel

Whatman 9cm GF/D glass microfibre filter

Sterile glass 800 ml bottle

Millipore XX6022050 Vacuum pump Millipore Corporation, Bedford, Massachusetts.

Sigmamotor TM5 peristaltic pump Middleport, New York.

Millipore Swinnex-47 autoclavable filter Millipore Corporation, Bedford Massachusetts.

250 ml conical flask

300μm coarse wire filter

Oxygen cylinder with gas flow valve and sterile tubing

0.22μm gas filter (COSTAR)

Thermostatically controlled Water bath SB 35 Grant Instruments Ltd Cambridge UK.

PERFUSION SOLUTIONS

Solution A

1000 ml of HBSS (Sigma, Poole, UK) (Calcium and magnesium free)

+10ml of HEPES (Sigma, Poole, UK) (10ml 1M HEPES/1000ml)

+10,000 U penicillin and 10,000 U streptomycin

pH corrected to 7.4 using sterile 1M hydrochloric acid and 0.1M sodium hydroxide

Solution B

600ml HBSS (Sigma, Poole, UK)

+0.5mM EGTA (Sigma, Poole, UK) (228.2 mg EGTA in 5 ml distilled water will require NaOH to dissolve (0.6mM/5ml) 2.5ml of EGTA solution added to 600ml HBSS)
+60,000 U penicillin and 60,000 U streptomycin

Solution C

600ml HBSS (Sigma, Poole, UK)
60,000 U penicillin and 60,000 U streptomycin

Enzyme Solution

300 ml HBSS (Sigma, Poole, UK)
0.05% Collagenase (type IV) (Sigma, Poole, UK) 150mg/300ml
0.017% Hyaluronidase (type IV-S) (Boehringer-Mannheim, Lewes, UK) 50mg/300ml
0.002% Deoxyribonucleic acidase (Boehringer-Mannheim, Lewes, UK) 6mg/300ml
5mM CaCl2 (1.5ml 1M solution in 300ml.)
50,000 U penicillin and 50,000 U streptomycin.

William’s Medium

500ml William’s Medium (GIBCO-BRL, Inchinan, UK)
50,000 U penicillin and 50,000 U streptomycin
5% heat inactivated foetal calf serum (ICN Flow biochemicals, UK)
2mM glutamine

Apparatus set up

The Buschner funnel was placed in a laminar airflow hood and taped to a universal container stand fixed to the bench. A Whatman glass fibre filter was placed inside the funnel. The outflow tubing of the funnel was connected to a sterile 3-way tap and connected in turn to tubing leading to the waste disposal container and to the perfusate bottle via the recirculating loop. The 3-way tap was set to allow flow to the waste disposal container. The vacuum tubing was connected to the waste disposal container using a 24Ch needle inserted through a hole bored in its cap. The perfusate bottle was connected to tubing leading to the peristaltic pump. The outflow tubing from
the peristaltic pump was connected via a Luer lock fitting to a sterile 3-way tap. One limb of the 3-way tap was connected via tubing to the perfusate bottle to allow recirculation and priming of the apparatus prior to perfusion, the other lead to the perfusing limb. The perfusing limb consisted of 4 3-way taps connected together by their male and female unions. The limb was supported using a retort stand and was connected to each of 3-4 short perfusing tubes which would later be connected to the cannulae.

Collection of specimens

Fresh specimens of liver weighing between 15 and 60g were obtained from patients undergoing hepatectomy. Specimens were immediately flushed with approximately 40 mls of heparinised 0.9% saline (500 units heparin per ml) at 4°C to remove blood clot form the specimen. Specimens were then placed in a sterile container containing iced/heparinised saline.

Preparation of specimen for perfusion

Specimens were placed on a sterile glass petri dish and were cannulated with 3-4 18Ch plastic cannulae (Venflon). Each cannula was sutured in place using 5/0 silk sutures (Ethicon, Edinburgh, UK). Following completion of cannulation remaining large vessels were sutured closed with 5/0 silk sutures. In specimens with multiple small vessels which could not be tied off individually the entire edge of the liver was oversewn with a continuous 5/0 silk suture. 2-3 stay sutures were placed at the edges of each specimen to allow movement of the specimen without dislodging the cannulae.

Perfusion Procedure

The liver specimen was placed on the Whatman filter in the Buschner funnel. The bottle containing Solution A was placed in the water bath and the cap with tubing was attached while maintaining sterility. Oxygen was infused through the solution at a
rate of 1 litre per minute for 2 minutes. The peristaltic pump was started at a flow rate of 50 ml per minute. The vacuum pump was connected to the waste disposal unit and started at a vacuum pressure of 5 bar. The 3 way tap on the perfusion circuit was initially set to recirculate and once air had been evacuated from the pump circuit the tap was switched to the perfusing limb. Perfusate was allowed to flow through each perfusing tube to expel air. The pump was then temporarily stopped and each perfusing tube was connected to its cannula. The pump was restarted and perfusion of the specimen commenced. At this point minor leaks from the liver surface could be visualised and if of sufficient size could be tied off using silk sutures. When 500 mls of the first bottle of Solution A had been perfused the pump was stopped and a second bottle of Solution A connected up. The waste disposal bottle was also replaced. Perfusion was then continued and an identical protocol was adopted with Solutions B and C.

Following perfusion with solution C the vacuum and peristaltic pumps were switched off. The 3-way tap on the waste disposal limb of the circuit was switched to allow recirculation back to the perfusate solution. The vacuum pipe was also connected to the perfusate bottle containing the enzyme solution. The vacuum and peristaltic pumps were then restarted and the enzymes allowed to recirculate for approximately 40 minutes. Following which the liver specimen developed signs of disaggregation.

Following enzymatic digestion the liver specimen was removed from the Buschner funnel and placed in a sterile petri dish. The cannulae were then removed and the silk sutures all removed to prevent them contaminating cultures. 20mls of supplemented William’s solution was added to the petri dish and the liver tissue was then teased apart. The hepatocytes could then be washed gently out of the specimen. The liver specimen was periodically transferred to a new petri dish and the William’s solution containing hepatocytes poured through the coarse mesh into a 250 ml conical flask. This process was repeated until approximately 200 mls of William’s and hepatocytes had been collected.
Figure 2.1a

Apparatus for liver perfusion
Purification of Hepatocytes

The cell suspension was then drawn up into a 50 ml syringe and discharged through a Millipore sv47 filter to remove larger clumps of cells. The cell suspension was transferred into 4 or 5 50 ml apex tubes and centrifuged at 500 rpm for 5 minutes. The supernatant was removed and the cell pellet resuspended in supplemented William’s medium. This process was repeated three times in total. After the final wash the cell pellets were resuspended in 10 mls of supplemented William’s solution and layered onto Ficol gradients which had been previously prepared. The gradients were placed in a centrifuge and spun at 2500 rpm for 20 minutes. Cells were harvested from the 40-60% interface as this was found to yield hepatocytes of high purity and high viability. Cell suspensions were then washed three times by suspension in William’s medium and centrifugation at 500 rpm for 10 minutes. Cells were made up to a final volume of 10mls. 100μl of the suspension was diluted 1:10 in 0.25 % trypan blue and counted in a counting chamber. Staining with the vital stain trypan blue permitted assessment of viability as viable cells exclude the dye whereas non-viable cells stain blue. Viability in excess of 95% was considered acceptable.

Preparation of tissue culture plates

Rat tail collagen (Boehringer Mannheim, Lewes, UK) was made up at a concentration of 66.7μg/ml in sterile distilled water containing 0.2% acetic acid. The solution was filtered using a 0.22μ filter immediately prior to use. The central 60 wells of high affinity 96 well tissue culture plates (Costar, High Wycombe, UK) were coated with rat tail collagen 30μl per well. Each well was visually examined to determine uniform coating of the base of the well. Plates were left uncovered in a laminar air flow hood until the collagen solution had evaporated to dryness. Plates were either used immediately or stored sealed and frozen at -70°C until required.
Plating of hepatocytes

Hepatocytes were diluted to a final concentration of $1.5 \times 10^5$ cells per ml and were then added to the central 60 wells of 96 well tissue culture plates which had previously been coated with rat tail collagen and air dried in the laminar airflow hood. 200µl of cells were added per well to give a cell count of $3 \times 10^4$ cells per well. Plates were covered and sealed using gas permeable insulation tape and were incubated at 37°C in 5% CO$_2$ and air in a humidified incubator for 24 hours. Following incubation plates were examined using an inverted microscope to ensure that hepatocytes had adhered to the base of the well, the supernatants were removed from each well and discarded. Each well was then gently washed by dribbling 200 µl of fresh medium into the well and aspirating it.

Hepatocyte culture

Following washing, cells were then ready for the addition of test substances. All test substances were made up in William’s medium supplemented with 5% foetal calf serum, 10,000 units of penicillin and streptomycin, 2mM glutamine. All cultures were performed over a 48 hour period with the plates being sealed with gas permeable tape and placed in a humidified incubator at a temperature of 37°C and 5% CO$_2$ and air mixture.

Hepatocyte viability

Cell viability was assessed using the crystal violet staining technique (Falconer et al, 1994). Briefly wells were coated with 100µl of 5% formalin in phosphate buffered saline for 10 minutes. Plates were then washed and allowed to dry completely prior to staining using 0.5% aqueous crystal violet for 10 minutes. Plates were then washed and allowed to dry prior to development using 100µl of 33% acetic acid per well. Plates were read at 570 nm using a MR5000 ELISA plate reader (Dynatech, Billinghurst, UK). The high purity of hepatocyte preparations was confirmed by flow cytometry.
(Beckton Dickinson, UK) and the cells were negative for CD14 (implying the near absence of monocytes and Kupffer cells).

Comment

Isolation of human hepatocytes is a demanding technique which is not widely practised. The methods of hepatocyte perfusion and isolation was originally based upon a technique described by (Berry et al, 1991) and refined by O'Riordain et al 1995 and later modified by the author in association with Dr J.A.Ross and Mrs J.P.Maingay. One of the major limitations to establishing a hepatocyte isolation programme is obtaining fresh liver tissue. The majority of the specimens perfused in the present study were provided by Mr O.J. Garden. Contamination of liver tissue with tumour is a concern and liver tissue was always obtained as far as possible from any tumour deposit. In addition no liver tissue was used from patients with multiple hepatic metastases and the patients who did undergo resection for malignancy all had solitary hepatocellular carcinoma or a solitary colorectal cancer metastasis. Activation of hepatocytes might occur in patients with chronic viral disease and no tissue was accepted from any patient with viral hepatitis. The effect of surgery on hepatocyte activation is uncertain. It is clear that the metabolic response to surgery has a rapid onset and since hepatic resection is a lengthy operation taking 3-4 hours it is conceivable that activation of hepatocytes might have occurred as a consequence of surgery itself. A study examining the effect of major and minor techniques of gallbladder surgery has demonstrated that elevation of serum IL-6 and CRP concentrations may occur within 1 hour of commencement of surgery (McMahon et al 1993, de Beaux et al 1995).

Hepatocytes are large polygonal cells which are therefore relatively easy to separate from other cells such as monocytes, fibroblasts and erythrocytes which might be present in the digested liver specimen. The initial digested product contains a large number of dead cells, other cells and debris and this was the reason for the lengthy purification process to obtain a relatively pure hepatocyte population. Flow cytometry
and microscopic examination confirmed the high purity of hepatocyte populations obtained in this way and exclusion of trypan blue confirmed viability.

In all of the present studies hepatocytes were cultured on a single layer membrane of rat-tail collagen. Hepatocytes do not bind well to plastic and some form of matrix is generally used to facilitate binding and adherence. Ryan et al, have demonstrated that for long term hepatocyte cultures a double layer collagen membrane which sandwiches the hepatocytes is the best method of maintaining hepatocyte protein synthetic function but that unilayer membranes are quite satisfactory for short term duration studies lasting less than 7 days.

The advantage of using human hepatocytes for type of experiment described in this thesis is that isolated human hepatocytes produce all of the acute phase proteins whereas human hepatoma cell lines do not. HepG2 cells do not produce C-reactive protein or alpha 1 antichymotrypsin but do produce alpha 1 acid glycoprotein and haptoglobin (Table 1.4). Furthermore the pattern of cytokine receptors expressed by isolated human hepatocytes is more likely to resemble that of Hepatocytes in vivo than would be the case for a hepatoma cell line. Finally short term primary cell cultures are less likely to be affected by contamination of cells by mycoplasma than are immortalised cell lines.

The principal disadvantage of using a primary culture of human hepatocytes is that cellular function inevitably deteriorates following isolation and this imposes a limitation on the period for which cells can be studied. The cumulative concentration of acute phase proteins increases over the first 96 hours of culture but the 24 hour production rate begins to decline after 72 hours (Table 2.1). In all of the experiments described in this thesis cells were plated for 24 hours following isolation and thus the period of study would be between 24 and 48 hours as indicated in the table.
Table 2.1

Production of acute phase proteins by isolated human hepatocytes. Results are expressed as ng/ml as a cumulative total (cumul) and as production occurring in each 24 hour (24hr) period.

<table>
<thead>
<tr>
<th>Time</th>
<th>C-reactive protein</th>
<th>α1-acid glycoprotein</th>
<th>Prealbumin</th>
<th>Transferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cumul 24 hr</td>
<td>Cumul 24 hr</td>
<td>Cumul 24 hr</td>
<td>Cumul 24 hr</td>
</tr>
<tr>
<td>24-48</td>
<td>100 100</td>
<td>341 341</td>
<td>34 34</td>
<td>166 166</td>
</tr>
<tr>
<td>48-72</td>
<td>152 52</td>
<td>568 227</td>
<td>57 23</td>
<td>266 100</td>
</tr>
<tr>
<td>72-96</td>
<td>187 35</td>
<td>712 144</td>
<td>57 0</td>
<td>272 6</td>
</tr>
</tbody>
</table>
Figure 2.1

Hepatic perfusion apparatus set to priming cycle to allow elimination of all air from the circuit prior to perfusion.
Figure 2.2

Hepatic perfusion apparatus set to perfusion cycle with collection of waste perfusate
Figure 2.3.
Hepatocyte perfusion apparatus set to recirculating loop used for enzymatic digestion of liver tissue.
ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Venous blood (20mls) was collected into a lithium heparin coated tube. The whole blood was carefully layered onto 20 mls of Ficoll (Percoll) 1077 in a 50ml apex tube. The tube was then centrifuged at 2,500rpm for 30 minutes. Following centrifugation discrete layers appeared with plasma uppermost then Ficoll and red cells in a pellet at the apex of the tube. The peripheral blood mononuclear cells being more dense than plasma but less dense than Ficoll separate into a discrete layer at the plasma / Ficoll interface. These cells were carefully removed by wide bore pipette and transferred to another apex tube. The cells were resuspended in 45 mls of RPMI medium and were centrifuged for a further 15 mins at 1800 rpm. The supernatant was then drawn off and the cells resuspended in a further 45 mls of RPMI. This was centrifuged at 1500 rpm for a further 15 minutes. The supernatant was withdrawn and the cells resuspended in 10 mls of RPMI medium. Cells were counted and diluted to a concentration of 1x10^6 cells per ml and plated in the central 60 wells of 96 well tissue culture plates (Costar, High Wycombe, UK, High, UK) at a concentration of 2x10^5 cells per well.

Stimulation of PBMC with lipopolysaccharide

In certain experiments PBMC were stimulated with lipopolysaccharide. The lipopolysaccharide used was E.coli 0.1027 and the concentration used was 10μg/ml. This concentration was chosen on the basis of preliminary experiments which demonstrated that this produced maximal stimulation of IL-6 and TNF production by PBMC isolated from healthy controls.
Table 2.2

Production of IL-6 and TNF by PBMC isolated from healthy control subjects \(n=12\) in the presence and absence of LPS 10\(\mu\)g/ml

<table>
<thead>
<tr>
<th></th>
<th>Unstimulated</th>
<th>Lipopolysaccharide 10(\mu)g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>1.1 ± 0.45</td>
<td>5.8±0.6</td>
</tr>
<tr>
<td>TNF</td>
<td>36.8± 7.6</td>
<td>1684 ±287</td>
</tr>
</tbody>
</table>

Stimulation of PBMC with phytohaemagglutinin.

Peripheral blood mononuclear cells were isolated as described above and plated in 100 \(\mu\)l of RPMI medium supplemented with penicillin and streptomycin in 96 well tissue culture plates 2x10^5 cells per well. Freeze dried phytohaemagglutinin (PHA) of reagent grade derived from Phaseolus spp was purchased from Murex Diagnostics Ltd, Dartford England. The PHA was reconstituted in 5 ml of sterile distilled water to make a stock solution of concentration 9mg/ml. 22 ml of RPMI medium containing penicillin and streptomycin was taken. 2.5ml of heat inactivated foetal calf serum and 0.5 ml of glutamine were added. 100ul of PHA stock solution was added to make a concentration of 100\(\mu\)g/ml.

PHA containing medium was added to cells at final concentrations of 0, 10 and 100\(\mu\)g/ml. Cells were incubated at 37°C in a humidified mixture of 5% CO2 and air for 72 hours. 100\(\mu\)l of tritiated \(^3\)H thymidine were added to 900\(\mu\)l of RPMI medium to make a solution containing 100\(\mu\) Ci. 10 ul or 1 \(\mu\)Ci were added to each well. cells were incubated for 2 hours at which point the medium was removed and the cells were stored frozen at -70°C until further analysis.
Measurement of $^3$H thymidine uptake

Plates were thawed and 200μl of phosphate buffered saline was added to each well. Cells were harvested using a Dynatech Mash Cell Harvester onto 10mm discs of glass fibre sheets. The discs were dried and added to scintillation vials to which 4ml of scintillant (Pico-fluor 15, Packard) was added. Vials were placed in an automated scintillation counter with positive and negative controls. Thymidine uptake was expressed in counts per minute and adjusted for degradation of the positive controls.

CYTOKINE ASSAYS

Measurement of IL-8, IL-6 and TNF concentrations in cell supernatants by ELISA

Measurement of IL-8, IL-6 and TNF in supernatants was performed by enzyme-linked immunosorbent assay (ELISA) (Goldie et al 1995). Briefly, for IL-6, microtitre plates (Costar, High Wycombe, UK) were coated with monoclonal anti-IL-6 antibody (Boehringer-Mannheim) or rabbit anti-human IL-8 (AMS Biotechnologies™, Witney UK) at 2μg/ml in 50mM NaCO3/NaHCO3 pH 9.6 for 4 hrs at room temperature. Samples were diluted 1/5 in 20mM Tris-buffered saline pH 7.4 containing 1% bovine serum albumin (TBS-BSA) and incubated at 4°C for 18 hours. The second antibodies were goat polyclonal anti-IL-6 (R&D Systems, Abingdon, UK) at 2μg/ml and goat anti-human IL-8 (R&D Systems, Abingdon, UK). These were detected with peroxidase-conjugated anti-goat IgG Fab fragments (Boehringer-Mannheim, Lewes, UK). For TNF, paired antibodies (Boehringer-Mannheim, Lewes, UK) were used in accordance with manufacturers instructions. Standard curves for each ELISA were constructed using recombinant human IL-8, IL-6 or TNF (R&D Systems, Abingdon, UK). The substrate was 3-3', 5-5'-tetramethylbenzidine in 100mM sodium acetate/citrate, pH 4.9. The reaction was stopped with 100μl of 1M sulphuric acid per well and plates were read at 490nm using a Dynatech MR5000 plate reader. Cytokine concentrations in samples were calculated from standard curves calculated using Assayzap computer software (Biosoft, Cambridge UK).
Table 2.3
Coefficients of variation (%CV) for measurement of IL-8, IL-6 and TNF and sensitivity by ELISA as described above.

<table>
<thead>
<tr>
<th></th>
<th>Intra assay %CV</th>
<th>Inter assay %CV</th>
<th>Sensitivity pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>5</td>
<td>11</td>
<td>190</td>
</tr>
<tr>
<td>IL-6</td>
<td>3</td>
<td>6</td>
<td>75</td>
</tr>
<tr>
<td>TNF</td>
<td>5</td>
<td>8</td>
<td>15</td>
</tr>
</tbody>
</table>

Measurement of IL-8 and IL-6 in serum was performed by enzyme-linked immunosorbent assay (ELISA). Briefly, for IL-6, microtitre plates (Costar, High Wycombe, UK) were coated with monoclonal anti-IL-6 antibody (Boehringer-Mannheim) or rabbit anti-human IL-8 (AMS Biotechnologies™, Witney UK) at 2μg/ml in 50mM NaCO₃/NaHCO₃ pH 9.6 for 4 hrs at room temperature. Samples were diluted 1/5 in 20mM Tris-buffered saline pH 7.4 containing 1% bovine serum albumin (TBS-BSA) and incubated at 4°C for 18 hours. The second antibodies were goat polyclonal anti-IL-6 (R&D Systems, Abingdon, UK) at 2μg/ml and goat anti-human IL-8 (R&D Systems, Abingdon, UK). These were detected with peroxidase-conjugated anti-goat IgG Fab fragments (Boehringer-Mannheim, Lewes, UK). For TNF, paired antibodies (Boehringer-Mannheim, Lewes, UK) were used in accordance with manufacturers instructions. Standard curves for each ELISA were constructed using recombinant human IL-8, IL-6 or TNF (R&D Systems, Abingdon, UK). The substrate was 3,3',5,5'-tetramethylbenzidine in 100mM sodium acetate/citrate, pH 4.9. The reaction was stopped with 100μl of 1M sulphuric acid per well and plates were read at 490nm using a Dynatech MR5000 plate reader. Cytokine concentrations in samples were calculated from standard curves calculated using Assayzap computer software (Biosoft, Cambridge UK). The IL-6 ELISA for measurement of serum IL-6 gave good reproducibility however the IL-8 assay for serum occasionally gave erratic results.
Measurement of acute phase proteins in hepatocyte supernatants was performed using a sandwich enzyme linked immunosorbent assay (ELISA). Briefly, 96 well plates (Costar, High Wycombe, UK) were coated with a 1:10,000 dilution of one of the following antibodies, goat anti-human CRP, goat anti-human α1-antichymotrypsin, goat anti-human transferrin, goat anti-human haptoglobin and goat anti-human prealbumin (Dako, UK). Plates were blocked with 1% bovine serum albumin. Supernatants were diluted 1:10 before addition to wells. Standard curves were constructed using human CRP calibrator (Sigma, Poole, UK) for CRP and human serum protein calibrator (Sigma, Poole, UK) for the other acute phase proteins. Following overnight incubation, plates were washed 4 times prior to the addition of a 1:5000 dilution of the following rabbit antibodies, anti-human CRP, anti-human α1-antichymotrypsin, anti-human transferrin, anti-human haptoglobin and anti-human prealbumin (Dako, High Wycombe, UK). These were detected by peroxidase-conjugated antibody directed against rabbit immunoglobulins at a dilution of 1:2000 (Sigma, Poole, UK) and the substrate 3,3',5,5'Tetramethylbenzidine. The plates were read at 490nm using a MR5000 ELISA plate reader (Dynatech, Billinghurst, UK) and concentrations in the samples were calculated using the AssayZap (Biosoft, Cambridge, UK.) computer software. The limit of sensitivity of each assay, taking into consideration the sample dilutions, was 120 pg/ml. The intra-assay and inter-assay coefficients of variation (%CV) of the ELISAs for each acute phase protein are shown in Table 2.4.
Table 2.4

Coefficients of variation for measurement of acute phase proteins in hepatocyte-supernatants by ELISA.

<table>
<thead>
<tr>
<th>Protein</th>
<th>intra-assay %CV</th>
<th>inter-assay %CVs</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein</td>
<td>3.01</td>
<td>7.8</td>
</tr>
<tr>
<td>haptoglobin</td>
<td>1.12</td>
<td>8.3</td>
</tr>
<tr>
<td>α1-antichymotrypsin</td>
<td>0.91</td>
<td>9.8</td>
</tr>
<tr>
<td>α1-acid glycoprotein</td>
<td>4.25</td>
<td>9.0</td>
</tr>
<tr>
<td>prealbumin</td>
<td>2.17</td>
<td>8.3</td>
</tr>
<tr>
<td>transferrin</td>
<td>1.66</td>
<td>7.6</td>
</tr>
</tbody>
</table>

*Measurement of serum C-reactive protein in sera*

The production by hepatocytes of C-reactive protein (CRP) was measured by sandwich ELISA (10). Briefly 96 well plates were coated with goat anti human CRP at a dilution of 1:5000. Plates were blocked using 1% BSA prior to washing. Sera were diluted 1:10,000 and were added to the coated plates. Standard curves were constructed using human C-reactive protein calibrator (Dako, Glostrup, Denmark). Samples and standards were incubated overnight and following washing, the second antibody, peroxidase conjugated anti-human CRP, was added. The substrate used was TMB and the reaction was stopped using 1M sulphuric acid. Plates were read at 490nm using a Dynatech MR5000 automated plate reader. Concentrations in samples were calculated from standard curves calculated using Assayzap computer software (Biosoft, Cambridge, UK). The lower limit of sensitivity taking into account sample dilutions was 100 μg/l.
Table 2.5

Coefficients of variation (%CV) and sensitivities of ELISA for measurement of serum C-reactive protein concentration.

<table>
<thead>
<tr>
<th></th>
<th>Intra assay %CV</th>
<th>Inter assay %CV</th>
<th>Sensitivity μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>7</td>
<td>15</td>
<td>100</td>
</tr>
</tbody>
</table>

In some studies the hospital laboratory measurements of serum C-reactive protein were used. These were based on an immunoturbidometric technique with a lower limit of sensitivity of 10 mg/l. Comparison of serum C-reactive protein concentrations measured by ELISA and by immunoturbidimetric assay demonstrated a significant correlation $R+0.93$ between the two techniques (Figure 2.4). Measurements by ELISA were however consistently greater by 30% than those measured by immunoturbidimetry. In addition the lower limit of detection of serum CRP by ELISA was an order of magnitude lower than that possible by immunoturbidimetry and values recorded as undetected by the latter techniques were not always undetected by ELISA.
Correlation between measurements of serum C-reactive protein by ELISA and by immunoturbidimetry. The light line indicates the expected correlation if measurements were equal using the two methods. The bold line indicates the actual correlation between measurement of CRP by the two methods.
CULTURE OF HUMAN PANCREATIC CANCER CELL LINES

Mycoplasma-free, adherent variants of the human pancreatic cancer cell lines MIA PaCa2, PANC 1 and CFPAC were obtained from the European tissue culture collection (Porton Down). Cells were grown in 75cm$^3$ flasks in RPMI supplemented with 5% foetal calf serum, 2mM glutamine and penicillin and streptomycin. The medium in which the cells were grown was changed approximately every 3 days. Cell cultures were split at weekly intervals or when this was required by cell overgrowth.

Splitting of cells: Medium was poured off the cells. 5 mls of trypsin solution containing EDTA was added and the cells rinsed to remove dead or loosely adherent cells. This trypsin was then removed and a further 5mls of fresh trypsin solution was added. Cells were then incubated for 10-20 minutes in a warm incubator until they could be easily detached by gently tapping the sidewall of the flask. The detached cells were transferred into a 20 ml universal container and 5 mls of supplemented RPMI was added. Cells were stained with trypan blue and counted. 1x10$^6$ cells were then returned to the culture flask and 20 mls of fresh RPMI medium was added to re-establish culture.
ANALYSIS OF PLASMA AND ERYTHROCYTE FATTY ACID COMPOSITION

Fatty acid analysis was performed by Professor M.J. Tisdale of the Department of Cancer Biochemistry, Pharmaceutical Sciences Institute, Aston University, Birmingham. Samples for plasma fatty acid composition analysis were drawn into 5ml vacuum tubes containing EDTA. Samples were immediately centrifuged at 2,500 rpm for 15 minutes. The plasma fraction was removed by pipette and stored at -70°C for subsequent analysis. The red cell fraction was washed 2 times by centrifugation with an equal volume of sterile phosphate buffered saline pH 7.4. Red cells were stored frozen at -70°C for subsequent analysis. Erythrocyte membranes were prepared by haemolysis of 200µl of erythrocytes in distilled water (Alexander et al, 1990). Samples were left on ice for 1 hour prior to centrifugation at 4,200 rpm for 15 minutes after which the supernatant was removed and discarded. The pellet was washed a further 4 times in 5 ml of PBS. Fatty acids were extracted from phospholipids by heating at 100°C for 45-60 minutes in 2.5ml of 5% sodium hydroxide in 50% methanol in an atmosphere of nitrogen (Folch et al, 1957). The solution contained butylated hydroxytoluene as an anti-oxidant and margaric acid as internal standard. The released fatty acids were methylated by heating to 80°C for 5 minutes in the presence of boron trifluoride in methanol. The fatty acid methyl esters were extracted twice with hexane:chloroform (4:1) and analysed using a Hewlett Packard 5890 Series II gas chromatograph, with a narrow bore (0.25mm) DB 32 column (J&W Scientific, Fisons, UK) and a flame ionisation detector at 250°C. The temperature programme ran for 28 minutes with an initial temperature of 180°C, with a 5°C/min increase to 220°C, which was then maintained for 15 minutes. The column was run with helium as the carrier gas. The fatty acids were identified from their retention times based on the use of authentic fatty acid methyl ester standards.
NUTRITIONAL ASSESSMENT TECHNIQUES

Anthropometry

Height was measured, with the patient standing upright in stockinged feet, to the nearest 0.1 cm using a wall mounted stadiometer. Pre-illness stable weight and duration of weight loss were recorded. Subjects were weighed on spring balance scales (Seca, Germany) without shoes and wearing light clothing. Ideal body weight was calculated using standardised tables (Metropolitan Life Assurance Tables). Mid upper-arm circumference was measured using tape measure to the nearest 0.1 cm at the midpoint between the olecranon and acromion processes. Mid-arm muscle circumference (MAMC) was calculated using Jelliffe’s equation. (Jelliffe 1966). Triceps skinfold thickness (TSF) was measured at the mid point between the olecranon and acromion processes overlying the triceps muscle using Harpenden callipers (Holtain Ltd, UK). Three measurements were performed and the mean value recorded.

Body composition analysis

Multiple frequency bioelectrical impedance analysis (MFBIA) (Xitron 4000 MFBIA Xitron Technologies, San Diego, CA, USA) operated at a current of 200μA root mean square was used to assess body composition. All values were recorded with the subject supine with limbs apart. Repeat measurements were performed using the same pair of limbs. Total body resistance and reactance were taken at 5, 50, 100, 200, 500 and 1000 KHz. Values for total and extracellular water spaces were obtained using equations validated in a similar patient group (Fearon et al, 1992; Hannan et al., 1994). Fat free mass (FFM) also termed lean body mass was calculated from total body water (TBW) assuming a constant hydration factor of 73.2% (Pace & Rathburn., 1945).

Resting Energy Expenditure.

Resting energy expenditure was measured by indirect calorimetry using a ventilated hood system (Deltatrac, S&W Vickers, UK). All recordings were conducted
in a thermoneutral environment between 8-9 am following an overnight fast with the patient lying supine and at rest. Prior to each measurement the equipment was calibrated using gas containing 95% oxygen and 5% carbon dioxide at a known barometric pressure. Flow through the canopy was kept constant at 44.3 l/min. Gas analysis was performed using a paramagnetic oxygen analyser and an infra-red carbon dioxide analyser. VO2 and VCO2 was measured over a 20 minute period and processed on line by a microprocessor and converted to mean energy expenditure using the abbreviated de Weir formula (de Weir., 1949). This system provides measurements of VO2 and VCO2 which have an error of less than 4% (Makita et al., 1990). Values are expressed per patient and in relation to total body weight and lean body mass. During the course of the study change in weight was incorporated, on an individual basis, in calculations of predicted energy expenditure and body composition analysis.

Assessment of nutritional intake

Food intake was recorded using a detailed four day food diary. Patients were requested to complete details of cooking method, or brand of prepared food product and to give weighed intakes wherever possible. Total calorific value of ingested food was calculated using Compeat 4 software (Nutrition Systems Ltd, London UK) and the mean value per day assessed. Calorific values were calculated by a contracted but independent operator experienced in dietary analysis tools. In addition patients were asked to score their current food intake compared to food intake prior to illness using a visual analogue scale.

Predictive equations for resting energy expenditure

Predicted resting energy expenditure was estimated using both the Harris-Benedict and Scholfield equations.
Harris Benedict equations: \( Wt = \) weight in kg, \( Ht = \) height in cm

Men REE (KCal) \[ = 66.5 + 13.75Wt + 5.00Ht - 6.77\text{age} \]

Women REE (KCal) \[ = 655.1 + 9.56Wt + 1.85Ht - 4.67\text{age} \]

Table 2.6

Scholfield equations: (Scholfield et al, 1985)

<table>
<thead>
<tr>
<th>Age</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-18</td>
<td>17.6wt + 656</td>
<td>13.3wt + 690</td>
</tr>
<tr>
<td>19-30</td>
<td>15.0wt + 690</td>
<td>14.8wt + 485</td>
</tr>
<tr>
<td>31-60</td>
<td>11.4wt + 870</td>
<td>8.1wt + 842</td>
</tr>
<tr>
<td>&gt;60</td>
<td>11.7wt + 585</td>
<td>9.0wt + 656</td>
</tr>
</tbody>
</table>

Good correlations were obtained between recorded resting energy expenditure and predictive equations for resting energy expenditure in patients with pancreatic cancer (Figure 2.5 &2.6). Predictive equations consistently over estimated REE in healthy control subjects and underestimated REE in patients with pancreatic cancer (Table 2.7 & 2.8). Recorded resting energy expenditure was significantly greater in patients with pancreatic cancer both as a total and expressed in relation to body weight compared with healthy controls.
Table 2.7

Resting energy expenditure recorded by indirect calorimetry and predicted resting energy expenditure (Harris-Benedict and Scholfield predictive equations), in healthy controls (n=17) and patients with pancreatic cancer (n=80).

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Pancreatic cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recorded resting energy expenditure (Kcal/24hrs)</td>
<td>1340 (1040-1890)</td>
<td>1430 (890-2550)</td>
</tr>
<tr>
<td>Harris-Benedict predicted REE (Kcal/24hrs)</td>
<td>1530 (1110-2030)(^a)</td>
<td>1300 (826-1919)(^c)</td>
</tr>
<tr>
<td>Scholfield predicted REE (Kcal/24hrs)</td>
<td>1600 (1189-2056)(^b)</td>
<td>1306 (989-1967)(^b)</td>
</tr>
</tbody>
</table>

Statistical significance compared with recorded energy expenditure for each group a, \(p<0.002\), b, \(p<0.0002\), c, \(p<0.0001\)

Table 2.8

Resting energy expenditure per kg body weight recorded by indirect calorimetry and predicted resting energy expenditure (Harris-Benedict and Scholfield predictive equations), in healthy controls (n=17) and patients with pancreatic cancer (n=80).

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Pancreatic cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recorded resting energy expenditure (Kcal/kg/24hrs)</td>
<td>18.9 (15.6-34.4)</td>
<td>24.2 (16.4-41.8)</td>
</tr>
<tr>
<td>Harris-Benedict predicted REE (Kcal/kg/24hrs)</td>
<td>21.8 (19.5-39.2)(^a)</td>
<td>22.1 (17.4-26.1)(^c)</td>
</tr>
<tr>
<td>Scholfield predicted REE (Kcal/kg/24hrs)</td>
<td>22.8 (18.2-33.7)(^b)</td>
<td>22.5 (17.4-30.3)(^b)</td>
</tr>
</tbody>
</table>

Statistical significance compared with recorded energy expenditure for each group a, \(p<0.002\), b, \(p<0.0002\), c, \(p<0.0001\) 2-tailed paired t-test.
Figure 2.5

Correlation between recorded resting energy expenditure (REE) and resting energy expenditure predicted using the Harris Benedict predictive equations in patients with pancreatic cancer (n=80). R=0.81, p<0.0001.
Correlation between recorded resting energy expenditure (REE) and resting energy expenditure predicted using the Scholfield predictive equations in patients with pancreatic cancer (n=80). R=0.79, p=0.0001.
Quality of Life

The importance of assessment of quality of life of cancer patients undergoing trial treatments has been increasingly recognized (Maguire & Selby, 1989; Selby et al, 1984). In this thesis a number of different tools were used to assess quality of life. The Karnofsky performance scale was used to assess status of patients who received either Megestrol acetate and ibuprofen (Chapter 9) and patients who received oral fish-oil supplementation (Chapter 10) (Karnofsky et al, 1948). In addition patients receiving megestrol acetate and/or ibuprofen were also assessed using the EuroQol EQ-5D questionnaire to assess their general health state and patients’ perception of their health (EuroQol Group, 1990). In the same study quality of life was also assessed using the 30 item questionnaire EORTC QLQ-C30. This tool has 6 functional scales (physical, role, emotional, cognitive, social, global health status) and several questions relating to a range of physical symptoms (Aaronson et al, 1993). The Rotterdam symptom score (de Haes et al, 1990) was used to assess physical and psychological morbidity in the trial of high purity eicosapentaenoic acid (Chapter 11). In addition the World Health Organisation 5 point scale was used to evaluate status for entry in to the trial of high purity eicosapentaenoic acid (WHO 1979).
Chapter 3

NUTRITIONAL STATUS OF PATIENTS WITH UNRESECTABLE PANCREATIC CANCER.
INTRODUCTION

One of the most distressing features of pancreatic cancer is marked and progressive weight loss (Falconer et al., 1995). Patients often report a decreased dietary intake which may be due to a combination of factors such as anorexia, early satiety, anxiety, pain, and nausea. Patients who develop a gastric outlet obstruction suffer from severe vomiting which may require surgery and this is almost inevitably ensued by significant weight loss. Some patients have significant malabsorption and require pancreatic replacement therapy, others develop diabetes which may be difficult to control (Permert et al., 1995). Furthermore, it has recently been demonstrated that patients with pancreatic cancer have an elevated resting metabolic rate compared with age and sex matched controls (Falconer et al., 1994). This phenomenon may further exacerbate the rate of weight loss observed in such patients. Nutritional depletion is associated with reduced resistance to infection, muscle weakness and impaired healing (Bistrian et al., 1975; Haydock & Hill, 1986; Jeejebhoy, 1986). Moreover, progressive weight loss has been associated with profound effects on psychological state (Larsson et al., 1995).

Despite the assertion in a number of reports that weight loss is a common feature of pancreatic malignancy (De Wys, 1986), little objective data is available describing the quantity and quality of such weight loss in patients with advanced malignancy. Clearly, prior to describing the mechanisms of weight loss in patients with cancer, it is necessary to attempt to define the population which is under investigation. In this study, 20 patients with histologically proven unresectable cancer of the pancreas underwent nutritional assessment at the time of their diagnosis and monthly thereafter until they were unable to attend follow-up (within two months of death). Weight has limitations as a marker of nutritional depletion and in order to identify the nature and extent of tissue loss in patients with cachexia it is important to define the components of body weight (Hill, 1988). Therefore, in addition to measurement of body weight,
upper arm anthropometry and bioelectrical impedance analysis were performed. A longitudinal study of weight loss and anthropometric changes experienced by a homogenous group of untreated patients with unresectable pancreatic cancer has not previously been reported.

PATIENTS AND METHODS

Subjects

Twenty patients with unresectable adenocarcinoma of the pancreas confirmed by histology were studied. The group comprised 12 men and 8 women of median age 60 years. None of the patients received either cytotoxic chemotherapy or radiotherapy but were given full supportive care. Prior to the study, relief of biliary or gastric outlet obstruction had been effected in 9 patients by palliative bypass surgery and in 10 by endoscopic insertion of a biliary stent. The following assessments were performed.

Assessment

At the initial visit pre-illness stable weight and duration of weight loss were documented. Recall weight loss was validated where possible by examination of patients records of unrelated previous attendance to hospital. Height was measured using a wall-mounted stadiometer, with the patients standing erect and without shoes. At each visit patients were weighed on spring balance scales (Seca, Germany). Mid upper arm circumference (MUAC) and triceps skinfold thickness (TSF) were measured as previously described (Chapter 2) and mid-arm muscle circumference (MAMC) was calculated using Jelliffe's equation. Actual values were expressed as a percentage of Jelliffe's Standards to provide an approximation of nutritional depletion (Jelliffe, 1966). At each visit patients underwent clinical examination and the presence of either ascites or peripheral oedema was recorded.
Body composition analysis

Bioelectrical impedance analysis was performed using a four terminal impedance analyser (BIA-101, RJL Systems Inc, USA). Electrodes were positioned on the right hand and foot, and the measurement was made with patients in supine position and with limbs slightly abducted from the body. Resistance (R) at 50kHz was recorded. Total body water (TBW) was calculated from equations previously derived on a heterogeneous surgical patient population which included a large proportion of cancer patients (Fearon et al, 1992). LBM and fat mass were calculated from TBW using established formulae (Elia, 1992).

Acute-phase protein response and plasma albumin

Serum concentration of C-reactive protein was measured using an immunoturbimetric assay (Abbott TDX, Abbott Laboratories, Maidenhead, UK). Serum albumin was measured using an automated bromocresol green dye binding technique.

Statistical analysis

Results for age are expressed as median and interquartile range. Comparison of data at different time points was performed using the Wilcoxon Sign Rank test.

RESULTS

Patient characteristics

The median age of patients was 60 years (range 42-83 years), 12 were male and 8 female. No patient had evidence of peripheral oedema or ascites at the time of diagnosis. By the time of the last visit 3 patients had clinical evidence of peripheral oedema and 2 had ascites.
**Percentage weight loss**

At the time of diagnosis the majority of patients: 17/20 (85%) had lost weight and prior to death all of the patients studied had lost weight. The median reported weight loss prior to diagnosis was 14.2% (10-20%) and the duration of this weight loss was 14 weeks (12-20). Over a median interval of 27 weeks (22.5-38.0) between the time of diagnosis and the patient's final assessment the percentage weight loss rose significantly to 24.5% (11.5-29.7) (p<0.0004). If a percentage weight loss >20% is assumed to indicate severe malnutrition 3/20 (15%) patients would be classified as severely malnourished at diagnosis whereas prior to death 12/20 (60%) has undergone severe weight loss (Windsor & Hill, 1988a).

Table 3.1

Weight, percentage weight loss and upper arm anthropometry at diagnosis and just prior to death (n=20)

<table>
<thead>
<tr>
<th></th>
<th>At diagnosis</th>
<th>Prior to death</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>53.2 (47.9-71.1)</td>
<td>49.4 (41.9-61.9)</td>
<td>0.0004</td>
</tr>
<tr>
<td>BMI</td>
<td>20.7 (19.5-23.6)</td>
<td>17.7 (16.6-23.1)</td>
<td>0.0003</td>
</tr>
<tr>
<td>PIWL</td>
<td>14.2 (10-20)</td>
<td>24.5 (11.5-29.7)</td>
<td>0.0004</td>
</tr>
<tr>
<td>TSF (mm)</td>
<td>10 (7.2-13.3)</td>
<td>5.7 (4.5-9.5)</td>
<td>0.0002</td>
</tr>
<tr>
<td>AMC (cm)</td>
<td>21.9 (19.9-24.6)</td>
<td>18.9 (16.6-21.8)</td>
<td>0.0003</td>
</tr>
</tbody>
</table>
Median and interquartile range, values at diagnosis vs death compared using a Wilcoxon signed ranks test.

**Body mass index**

The median recalled pre-illness stable weight was 63.7kg (52.4-84) giving a pre-illness BMI at the upper end of the normal range: 24.9 (22.4-27.4) (Table 3.1). By the time of diagnosis patients had lost a median of 14.2% of their pre-illness stable weight and their median BMI was significantly reduced to 20.7kg/m² (19.5-23.6) (p<0.002 vs pre-illness BMI). During the course of the study further weight loss occurred such that by the time of death patients had lost 24.5% of their pre-illness stable weight and had a BMI of 17.7 kg/m² (7.2-13.3), while just prior to death the median BMI had decreased further to 17.7 (16.6-23.1) (p<0.0003). The median amount of weight lost by the time of diagnosis was 9kgs (5.5-12.5) and between diagnosis and death patients lost a further 5kgs (3.6-7.9), a total decrease of 14kgs (Figure 3.1). At diagnosis 7/20 (35%) patients had a BMI<20 and would be classified as underweight (Garrow, 1988) whereas at the time of death the majority of patients (13/20 ie 65%) had a BMI of <20.

**Arm Muscle Circumference**

At the time of diagnosis the median AMC was 21.9 cm (19.9-24.6) representing a value of 91.5% ideal (84.5-99.5). Just prior to death the median AMC had dropped significantly to 18.9cm (16.6-21.8) representing 77% ideal (70.5-90) (p<0.0003) (Table 3.1). The median decline in AMC between diagnosis and death was 2.1cm (1.2-5.2). If it is assumed that an AMC <85% of ideal indicates malnutrition the percentage of patients who would be classified as malnourished increases from 30% at the time of diagnosis to 70% just prior to death (Gray & Gray, 1979).
Figure 3.1

Weight loss as a percentage of pre-illness stable weight in 20 patients with pancreatic cancer. Percentage weight loss between onset of weight loss and the time of diagnosis is indicated by the broken line. Subsequent percentage weight loss based on clinical measurements is indicated by the solid boxes and lines. Vertical bars indicate interquartile ranges.
Triceps Skinfold thickness

At the time of diagnosis the median TSF was 10.0mm (7.2-13.3) representing a value of 70% ideal (53-83.5). Just prior to death the median TSF had reduced significantly to 5.7mm (4.5-9.5)) representing 43% ideal (31-69.5) (p<0.0003). The median decrease in TSF between diagnosis and death was 2.1mm (0.9-4.9). If it is assumed that a TSF <80% of ideal indicates malnutrition the percentage of patients who would be classified as malnourished increases from 65% at the time of diagnosis to 90% just prior to death (Gray & Gray, 1979).

Body composition at diagnosis and death

Bioelectrical impedance analysis demonstrated a significant decline in both fat mass and LBM between the time of diagnosis and death (Table 3.2). The median loss of LBM was 2.9kg (1.6-7.2) and of fat mass 2.7kg (0.9-4.4). At diagnosis no patients had clinical evidence of peripheral oedema or ascites. The median TBW content in this group was 31.7 L (26.9-38.9) representing 55.3% of body weight and in the middle of within the normal range ie 50-60% of body weight. Prior to death the TBW had decreased to 29.3L (24.5-37.0) (p<0.008) presumably due to an overall decrease in body weight (see Table 3.1). As a percentage of body weight TBW increased from 55.3% (51.8-59.9) to 59.1% (55.6-62.2), however this was not statistically significant. At the time of death 3 patients had clinical evidence of peripheral oedema and 2 had ascites.
Table 3.2
Body composition, albumin and C-reactive protein at diagnosis and prior to death (n=20)

<table>
<thead>
<tr>
<th></th>
<th>At diagnosis</th>
<th>Prior to death</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBM (kg)</td>
<td>43.4 (36.8-53.30)</td>
<td>40.1 (33.5-50.7)</td>
<td>0.03</td>
</tr>
<tr>
<td>Fat Mass (kg)</td>
<td>15.5 (11.4-19.9)</td>
<td>10.2 (6.4-14.9)</td>
<td>0.08</td>
</tr>
<tr>
<td>TBW (L)</td>
<td>40.0 (36.5-43.4)</td>
<td>38 (32.2-41.4)</td>
<td>0.03</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>10 (10-50)</td>
<td>35 (7-66)</td>
<td>0.006</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>42 (38-46)</td>
<td>34.5 (29-37)</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

Median and interquartile range, values at diagnosis vs death compared using a Wilcoxon signed rank test.
DISCUSSION

In this study progressive changes in nutritional status have been investigated in a group of patients with pancreatic cancer from before the time of diagnosis to a time point close to death. Body weight is the most commonly used indicator of nutritional status in the UK and is readily obtainable in the setting of an out-patient clinic (Payne-James et al, 1992). In order to take account of height BMI was also calculated. BMI has limitations as a nutritional assessment tool in the elderly since osteoporosis and consequent reduction of vertebral column length may result in underestimation of height and overestimation of BMI. In the present study the median age of the population was 60 years and osteoporosis was not a problem and since the potential error of BMI is one of overestimation it was considered acceptable to use BMI as an index. This study has demonstrated that patients with pancreatic cancer have lost approximately 15% of their pre-illness stable weight by the time of diagnosis and that this weight loss continues to progress with a median weight loss of 25% of pre-illness stable weight by the time of death. Using a weight loss >20% to signify severe malnutrition, 15% of patients in this study would be classified as severely malnourished at diagnosis while just prior to death this proportion had increased to 60%. Similarly, using a BMI of <20 kg/m² to signify malnutrition, 35% of patients were malnourished at the time of diagnosis increasing to 65% just prior to death.

The principal disadvantage of weight and BMI as indicators of nutritional status are that they do not provide specific information on the nature of tissue loss. In malnourished, hypoalbuminaemic or metabolically stressed individuals, weight is often influenced by oedema and therefore weight or BMI will tend to underestimate nutritional depletion in such patients (Barac-Nieto et al, 1978; Starker et al 1985). In this study subcutaneous fat and skeletal muscle protein were estimated by anthropometry and body composition was measured by bioelectrical impedance analysis. Significant reduction of arm muscle circumference was observed such that at
the time of diagnosis 30% of patients had significant arm muscle protein depletion (i.e. <85% of standardised reference values) and by the time of death this proportion had risen to 70% of patients. Similarly, depletion of subcutaneous fat, which represents 20-75% of total fat stores, was observed. Using a TSF <80% to signify malnutrition 55% of patients would be classified as significantly malnourished at diagnosis and 90% just prior to death. These data confirm previous detailed studies of body composition in the cancer patient, which have demonstrated that the principal tissues depleted are skeletal muscle and fat (Fearon & Preston, 1990).

At diagnosis the median TBW content was approximately 32L representing 55% of body weight and was within the normal range (50-60%). At this time no patient had clinical evidence of peripheral oedema or ascites. Just prior to death the TBW content had decreased significantly to 29 L presumably due to an overall decrease in body weight. As a percentage of total weight TBW increased from 55% to 59% however this difference was not statistically significant. It is known that starvation causes the body to retain sodium and water, hence the chronically starved subject often shows less weight loss than expected (Boulter et al, 1973). The present study suggests that patients with pancreatic cancer exhibit a state that is characterised by hypoalbuminaemia with a trend towards relative expansion of total body water space. Detailed body composition analysis in cachectic patients with lung cancer has suggested that this phenomenon may be explained by maintenance of total body water despite depletion of fat and muscle protein stores (Fearon & Preston, 1990). This picture of relative (but not absolute) expansion of the TBW combined with hypoalbuminaemia may or may not be associated with clinical signs of fluid retention such as oedema or ascites. In patients who do not have peritoneal carcinomatosis, clinically obvious signs of fluid retention such as oedema or ascites tend not to be apparent until a very late stage in the natural history of the disease, indeed in the present study at the time of the final assessment only 5 patients had clinical evidence of peripheral oedema or ascites.
Progressive nutritional depletion is a source of considerable distress and anxiety to patients with pancreatic cancer. In addition, it may have significant implications for their duration and quality of life (Inagaki et al, 1974). The physiological impairments and ultimately mortality associated with undernutrition are related to a loss of total body protein and this is reflected by the high incidence of hypostatic pneumonia as the terminal event in the starving patient (Moore, 1980). Windsor & Hill (1988b) have shown that patients with more than 15% weight loss are likely to have clinically significant (i.e. >20%) loss of body protein. At this level of protein depletion they have shown that physiological function, including for example respiratory muscle function, is significantly impaired. During the course of the present study the degree of weight loss observed was such that the majority of patients would be included in this high risk category.

This study indicates that nutritional depletion is already established by the time of diagnosis in the majority of patients with pancreatic cancer and that untreated, it will continue to a point where protein depletion is so marked that complications of starvation and impaired muscle physiology are likely. Cachexia associated with malignancy has long been a therapeutic target however few approaches have resulted in gain of lean tissue. Therefore to achieve effective therapeutic intervention we must understand the wasting process. Studies which have targeted impaired protein-energy intake by means of enteral or parenteral hyperalimentation have been disappointing (Cohn et al, 1982; Bozetti, 1992). These studies have demonstrated that when weight is gained it is usually as a consequence of an increase in total body water and fat rather than gain in skeletal muscle protein. Under these circumstances nutritional support is unlikely to be effective in reducing morbidity and mortality. Kern and Norten (1988) have indicated that therapeutic intervention in cancer cachexia must address both protein-energy deficit and the underlying metabolic derangements which prevent effective utilisation of nutrients. The inflammatory response to a tumour may be a key...
factor leading to altered host metabolism. An investigation was therefore undertaken to define the magnitude of energy deficits in patients with pancreatic cancer and to establish whether a relationship exists between the acute phase protein response and energy balance in weight-losing patients with pancreatic cancer.
Chapter 4.

NUTRITIONAL INTAKES AND THE CONTRIBUTION OF ANOREXIA AND HYPERMETABOLISM TO ENERGY DEFICIT IN PATIENTS WITH PANCREATIC CANCER
INTRODUCTION

Pancreatic cancer is frequently associated with cachexia, as demonstrated in Chapter 3, however, the mechanisms of weight loss are unclear. Weight loss is generally associated with a negative energy balance which can result from a rise in energy expenditure, a fall in energy intake or a combination of the two. Both sides of the energy balance equation can be influenced by activation of the pro-inflammatory cytokine network and excess production of pro-inflammatory cytokines has been shown to result in weight loss in animal models of cancer cachexia. Administration of intravenous TNF in rats is associated with weight loss accounted for largely by reduction in nutritional intake (Tracey et al, 1988). Cytokines are thought to suppress hypothalamic appetite regulation via a central action (Laviano et al, 1995). Animals which have been passively immunised with anti-TNF antibody remain weight stable and have normal dietary intakes when TNF is administered (Smith & Kluger, 1993). The role of cytokines in the regulation of human appetite is uncertain (Laviano et al, 1996). A clinical trial using the TNF antagonist pentoxyfilline failed to demonstrate a clinical benefit, however in that study the authors did not demonstrate a primary effect of pentoxyfilline on TNF pathways at the dose tested (Goldberg et al, 1995).

It has previously been demonstrated that weight losing patients with pancreatic cancer frequently have increased resting energy expenditure (Falconer et al, 1994). In such patients, energy expenditure was greatest in those patients with evidence of another cytokine-mediated metabolic event, namely an acute phase protein response (APPR). The aim of the present study was to analyse energy balance in a group of untreated patients with pancreatic cancer and to determine if an association existed between the presence of an acute phase response and a reduced food intake. One factor which is thought to exacerbate anorexia in patients with pancreatic malignancy is hyperbilirubinaemia secondary to obstructive jaundice (Fahie et al, 1995). All patients with obstructive jaundice were therefore excluded from this study.
PATIENTS AND METHODS

Patients

Thirty five patients with a recent diagnosis of adenocarcinoma of the pancreas were studied. No patient had undergone any surgical or endoscopic procedure in the preceding 4 weeks. No patient had received chemotherapy, radiotherapy or specific nutritional advice however full supportive care was given.

Food intake estimation

A variety of clinical tools have been devised to measure food intake. Accurate estimation of food intake depends on sampling a representative period of eating and on patient compliance in accurately recording intake. Assessments using 24 hour recall or food diaries of less than 4 days are thought to be inaccurate. Although longer term recording of food intake is more accurate diaries compiled for periods greater than a week can be intrusive to patients and depend on continuing patient co-operation to complete them accurately. In the present study food intake was documented using a detailed 4 day food diary including weighed intakes. Patients were asked to report precise details of food including specific brands and full details of food preparation and method of cooking. Data was analysed using Compeat 4 (Nutrition Systems Ltd, London) software by an independent observer.

Nutritional assessment

Patient’s weight, pre-illness stable weight and duration of weight loss were recorded. Recall weights were verified from hospital records where possible. Resting energy expenditure and total body water were measured by indirect calorimetry and multifrequency bioelectrical impedance analysis as described previously (Chapter 2). Predicted resting energy expenditure was calculated using the Schofield equations (Chapter 2). Total energy expenditure was calculated as the product of reported physical activity level and resting energy expenditure. The calorific value of lean tissue lost was
calculated with the assumption that all tissue lost was lean tissue and on the basis of adipose tissue having a calorific value of 9000KCal/kg. Energy deficit was calculated per day by dividing the total calorific value of tissue lost by duration of weight loss. The component parts of this energy deficit were calculated as follows:

\[ \text{dietary deficit} = \text{recommended nutritional intake} - \text{recorded nutritional intake} \]

and

\[ \text{energy expenditure deficit} = \text{predicted resting energy expenditure (Schofield)} - \text{recorded resting energy expenditure}. \]

**Serum C-reactive protein and albumin estimation**

Serum concentrations of C-reactive protein were measured by enzyme linked immunosorbert assay as described previously and albumin was measured by binding to bromocresol green as also described previously.

**Statistics**

To evaluate the possible influence of the acute phase response on energy balance, patients were divided into those with and without an acute phase response on the basis of serum CRP concentration being greater than or equal to 50mg/l. Statistical analysis was performed using the Mann Whitney U test.
RESULTS

The characteristics of the study group are given in Table 4.1. There were 21 males and 14 females of median age 65 years. The majority of patients had advanced malignancy with 77% having stage 3 or stage 4 tumours.

Table 4.1
Patient characteristics of 35 patients with pancreatic cancer undergoing analysis of dietary intake and measurement of energy expenditure

<table>
<thead>
<tr>
<th>Age</th>
<th>65 (56-71)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M:F</td>
<td>21:14</td>
</tr>
<tr>
<td>Stage</td>
<td>2:1:2:1</td>
</tr>
</tbody>
</table>

Nutritional intakes

The median energy intake of patients was 1702 (1420-2200) KCal/day. This represented 82% (73-92) of the recommended nutritional intake for a healthy age and sex matched UK population. When energy intake was broken down into its components (Figure 4.1) there were no significant percentage differences in source of energy compared with dietary reference values. Similarly, vitamin (Figure 4.2) and mineral (Figure 4.3) intakes were all greater in cancer patients than lower reference nutrient intakes for healthy age and sex matched UK populations. Dietary intakes of individual amino acids (Figure 4.4) and fatty acids (Figure 4.5) could not be compared with reference populations since no minimum requirements have been established for these substrates.

Energy expenditure

The median value of estimated resting energy expenditure using the predictive Schofield equations was 1305 (1198-1476) KCal/day (Table 4.2). The median recorded resting energy expenditure was 1510 (1415-1688) KCal/day and this value was
significantly greater than the predicted value (p<0.003) (Table 4.2). Total energy expenditure is calculated from the product of physical activity index and resting energy expenditure. The median physical activity index was 1.3 (1.2-1.5) and this gave a calculated total energy expenditure of 1963 (1840-2130) KCal/day (Table 4.2).

Table 4.2

Weight loss, energy expenditure and nutritional intakes of patients with pancreatic cancer (n=35)

<table>
<thead>
<tr>
<th>Weight (kg)</th>
<th>62 (53-70)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-illness stable weight (kg)</td>
<td>75 (61-83)</td>
</tr>
<tr>
<td>Weight loss (kg)</td>
<td>8.4 (5.3-17)</td>
</tr>
<tr>
<td>Duration of weight loss (weeks)</td>
<td>24 (16-32)</td>
</tr>
<tr>
<td>Calorific value of lost tissue (KCal)</td>
<td>56000 (36750-117250)</td>
</tr>
<tr>
<td>Energy deficit required to account for lost tissue (KCal/day)</td>
<td>425 (254-643)</td>
</tr>
<tr>
<td>Predicted REE (KCal/day)</td>
<td>1305 (1198-1476)</td>
</tr>
<tr>
<td>Recorded REE (KCal/day)</td>
<td>1510 (1415-1638)</td>
</tr>
<tr>
<td>Energy intake (KCal/day)</td>
<td>1709 (1420-2200)</td>
</tr>
<tr>
<td>Estimated total energy expenditure (KCal/day)</td>
<td>1963 (1840-2129)</td>
</tr>
<tr>
<td>Recorded-pred REE (Kcal/day)</td>
<td>142 (40-234)</td>
</tr>
<tr>
<td>Total energy expenditure -nutritional intake (KCal/day)</td>
<td>343 (-263-459)</td>
</tr>
</tbody>
</table>
Figure 4.1
Nutrient intake in patients with pancreatic cancer (n=35) and dietary reference values for carbohydrate, fat, protein and alcohol as a percentage of daily total energy intake (percent of food energy).
Figure 4.2
Vitamin intakes of patients with pancreatic cancer (n=35) and lower reference nutrient intakes of the UK population.

Vitamin E
vitamin D*
vitamin C
Biotin
Pantothenic acid
Folate
vitamin B12*
vitamin B6
Niacin
riboflavin B2
thiamin B1
vitamin A

mg/day (*=μg/day)

Lower reference intake
Recorded in
Figure 4.3

Dietary intakes of minerals of patients with pancreatic cancer (n=35) and lower dietary reference values of the UK population.
Figure 4.4

Amino acid intakes of patients with pancreatic cancer (n=35)
Dietary intake of monounsaturated, polyunsaturated and saturated fatty acids in patients with pancreatic cancer (n=35)
Calculation of energy deficits

The energy deficit attributable to hypermetabolism was calculated by subtracting predicted resting energy expenditure from recorded REE and was 142 (40-234) KCal/day (Table 4.2). The energy deficit attributable to reduced nutritional intake was calculated by subtracting energy intake from predicted total energy expenditure and was 343 (-263-459) KCal/day (Table 4.2).

Serum C-reactive protein and albumin concentrations

The median serum CRP concentration measured was 26mg/l range <10-67.5. Elevated serum CRP (>10mg/l) was detected in 19/35 patients (54%) and were not elevated in 16/35 (46% of patients). The median serum albumin concentration was 37 g/l (range 35-39).

Energy balance in patients with pancreatic cancer in relation to the presence of serum acute phase protein concentration

Patients were subdivided into 16 without an APPR (CRP<10 mg/l) and 19 with an APPR (CRP>10 mg/l). The patients were matched for age, weight, predicted REE and estimated total energy expenditure. Patients with an APPR had a significantly greater rate of weight loss than patients without an APPR (2.3 (1.4-3.3) vs. 1.1 (0.8-2.4) kg/month; p<0.02) (Table 4.3). In addition resting energy expenditure was significantly higher in patients with an APPR compared with those without an APPR (26.6 (25-29.9) vs. 23.3 (19.8-25.2) KCal/kg/day; p<0.002). Similarly energy intake was lower in patients with an APPR compared with those without an APPR (1553 (1355-1783) VS. 2192 (1517-2487) KCal/day; p<0.05).
Table 4.3

Energy balance in pancreatic cancer cachexia

<table>
<thead>
<tr>
<th></th>
<th>CRP &lt;10 mg/l (n=16)</th>
<th>CRP &gt;10 mg/l (n=19)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66 (54-72)</td>
<td>65 (57-69)</td>
<td>ns</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>67.5 (59-78)</td>
<td>60 (53-66)</td>
<td>ns</td>
</tr>
<tr>
<td>Rate of weight loss (kg/month)</td>
<td>1.1 (0.8-2.4)</td>
<td>2.3 (1.4-3.3)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Predicted REE (KCal/kg/day)</td>
<td>21.9 (19-24.3)</td>
<td>21.6 (21-25)</td>
<td>ns</td>
</tr>
<tr>
<td>Recorded REE (KCal/kg/day)</td>
<td>23.3 (19.8-25.2)</td>
<td>26.6 (25-29.9)</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Estimated total energy expenditure (KCal)</td>
<td>2272 (1768-2408)</td>
<td>2265 (2059-2471)</td>
<td>ns</td>
</tr>
<tr>
<td>Energy intake (KCal)</td>
<td>2192 (1517-2487)</td>
<td>1553 (1355-1783)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total energy deficit</td>
<td>-119 (-419-110)</td>
<td>-589 (-1113--370)</td>
<td>&lt;0.002</td>
</tr>
</tbody>
</table>

Results are medians and interquartile ranges. Statistical analysis was performed using the Mann Whitney-U test. Results outwith 95 per cent confidence limits were considered not significant (ns).

Associations between the APPR and energy intake and resting energy expenditure

A significant correlation was observed between serum CRP concentration and the magnitude of energy deficit due to reduced energy intake (R=0.43, p<0.01) Figure 4.6. A significant association was similarly demonstrated between serum CRP concentration and magnitude of energy deficit due to elevated resting energy expenditure (R=0.15, p<0.05) Figure 4.7. The distributions of energy deficits due to reduced energy intake and increased energy expenditure are illustrated for patients with and without an acute phase protein response in Figure 4.8. This plot demonstrates that in general terms patients without an elevated CRP tend to be clustered close to the intersection of the x and y axes indicating a normal energy distribution whereas patients with a elevated CRP are distributed along or between the x and y axes indicating greater energy deficits. Among patients with an elevated CRP considerable inter-individual variation of energy deficits is evident.
Figure 4.6

Correlation between serum C-reactive protein and energy deficit due to reduced nutritional intake in patients with pancreatic cancer (n=35). (R=0.43, p<0.01 regression analysis and analysis of variance)
Figure 4.7

Correlation between energy deficit due to hypermetabolism and serum C-reactive protein concentrations in patients with pancreatic cancer (n=35). (R=0.15, p<0.05 regression analysis and analysis of variance)
Figure 4.8

Distribution of energy deficit in 19 patients with pancreatic cancer who had an acute phase protein response (CRP>10 mg/l) and 16 patients who did not have an ongoing APPR.
DISCUSSION

In this study the energy intake of patients was significantly reduced compared with recommended nutritional intakes. Further analysis demonstrated that the proportion of the energy sources fat, carbohydrate and protein were comparable with an age matched population (Figure 4.1). This suggests a uniform reduction in total food intake rather than avoidance or deficiency of a specific dietary component. Similarly, despite having reduced calorific intake there was no evidence of reduction of vitamin or mineral intakes below recommended daily intakes (Figures 4.2 & 4.3). Energy intake was significantly lower in patients with an APPR than in those without and accounted for a greater proportion of the calculated total energy deficit. A significant correlation was observed between serum CRP and the energy deficit calculated to be attributable to a reduced nutritional intake. Since anorexia is the principal factor determining dietary intake in patients with gastrointestinal malignancy (in the absence of intestinal obstruction or osychogenic nausea and vomiting) it could be postulated that the correlation between the presence of an APPR (a known cytokine-mediated event) and reduced dietary intake, may imply a cytokine-mediated suppression of appetite regulation in this group.

Energy intakes did not meet energy requirements i.e. total energy expenditure. Indeed a deliberately low estimate was taken of physical activity index in order to underestimate rather than over estimate total energy expenditure. Total energy expenditure values for an age and sex matched healthy population of 2500KCal per day are usually described (estimated TEE in this study 1963 (1840-2129) KCal/day). Recorded REE was significantly greater in patients than predicted values demonstrating the prevalence of accelerated metabolism in this patient population. When patients were subdivided on the basis of an APPR in both groups recorded REE was significantly greater than predicted values. However, this difference was greatest in those patients with an APPR. Furthermore recorded REE was significantly greater in the patients with
an APPR than in the patients without an APPR. This observation presumably lead to the significant correlation between serum CRP and energy deficit due to hypermetabolism (Figure 4.6).

When energy deficits attributable to increased energy expenditure and reduced energy intake were correlated, it was evident that patients with an APPR had a higher incidence of both reduced intake and increased REE and that the degree of abnormalities of energy balance were more marked than in patients who did not have an ongoing APPR. It should be noted however that large inter-individual variations in the proportion of energy deficit due to reduced nutritional intake and increased energy expenditure existed both in patients with and without an elevated APPR.

Acute phase protein production is thought to be regulated principally by pro-inflammatory cytokines such as interleukin-6 and tumour necrosis factor-α and TNF has established effects on central appetite regulation. In this study the presence of the cytokine mediated APPR was associated with reduced dietary intake and increased REE. The APPR may therefore be a valid therapeutic target and the origin of the APPR in pancreatic malignancy required investigation prior to developing therapeutic strategies. Increased production of IL-6 and TNF has been previously described in weight-losing patients with pancreatic cancer. Further studies were therefore undertaken to investigate the possible origin of the APPR initially, by studying the production of proinflammatory cytokines by peripheral blood mononuclear cells isolated form patients with pancreatic cancer and by evaluating the effects of such cytokines on acute phase protein production by isolated human hepatocytes.
Chapter 5.

PRO-INFLAMMATORY CYTOKINE PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS ISOLATED FROM PATIENTS WITH CANCER, MULTIPLE ORGAN FAILURE AND HEALTHY CONTROLS AND THE ACUTE PHASE RESPONSE IN VITRO.
INTRODUCTION

The acute phase response is a complex physiological event which forms part of the metabolic response to acute stress or injury and which is also associated with chronic inflammatory disease. An important component of the acute phase response involves reprioritisation of hepatic protein synthesis wherein there is increased synthesis of the positive acute phase proteins, which in man include Serum amyloid A, C-reactive protein, α1-acid glycoprotein, α1-antichymotrypsin and haptoglobin and decreased synthesis of normal export proteins notably albumin, prealbumin and transferrin (negative acute phase proteins) (Kushner). In the previous chapter the presence of an elevated acute phase protein response (APPR) was found to correlate with increased resting energy expenditure and reduced energy intake in weight losing patients with pancreatic cancer. Central to the initiation of APPR in man is the local and systemic production of pro-inflammatory cytokines notably interleukin-6, tumour necrosis factor α and interleukin-1. IL-6, which is principally produced by cells of the macrophage/monocyte series, has been shown to stimulate the production of intracellular messengers which interact with the CRP nuclear promoter (Majello et al, 1990) and other promoters which are involved in the transcriptional activation (Morrone et al, 1988) of a number of acute phase proteins. In vitro the effects of IL-6 as the principal regulator of the acute phase response are modified by the presence of other cytokines including TNF, IL-1, IL-11, IL-13 oncostatin M and LIF and also by counter regulatory hormones insulin, adrenaline and the glucocorticoids (O’Riordan et al, 1995).

The acute phase proteins are thought to have an important role as opsonins, facilitating phagocytosis of bacteria and dead or damaged cells, as anti-proteases limiting tissue damage and as transport proteins (Kushner et al, 1989). There is evidence to suggest, however, that under certain circumstances the intensity or prolonged duration of activation of the acute phase response may be detrimental to the host (Falconer et al, 1995, Reeds et al, 1994). Amino acids required for acute phase protein synthesis are thought to be derived by accelerated breakdown of skeletal muscle (Reeds
et al, 1994). Patients with sepsis and multiple organ failure, who are known to undergo profound skeletal muscle catabolism, have been shown to have an elevated cytokine and acute phase protein response (Goldie et al, 1995). Similarly weight-losing patients with gastrointestinal malignancy also have augmented production of IL-6 and TNF by peripheral blood mononuclear cells (PBMC) in vitro and this has been shown to correlate with the presence of elevated serum C-reactive protein concentration (Falconer et al, 1994).

T-lymphocyte derived cytokines such as Interleukin 4 and interleukin 10 have been shown to down regulate production of IL-6 and TNF by peripheral blood mononuclear cells (Essner et al, 1994, Lee et al, 1990, Fiorentino et al, 1991) and to alter the phenotype of such cells (teVelde, 1988). It has been proposed that these lymphokines may form a homeostatic mechanism regulating pro-inflammatory cytokine production by monocytes. It has been demonstrated that T cell function is impaired in patients with gastrointestinal malignancy (Monson et al, 1990) and multiple organ failure. One such manifestation of impaired T-cell function is the decreased production of IL-2. IL-2 is the principal stimulator of IL-4 and IL-10 production by human lymphocytes and as such can be considered to be the principal regulator of T-lymphocyte function. Since IL-4 and IL-10 suppress pro-inflammatory cytokine production, failure of T-cell mediated regulation of pro-inflammatory cytokine release might therefore contribute to abnormal control of the acute phase response in disease. It is possible therefore that enhancing the IL-4 mediated suppression of pro-inflammatory cytokine production might be of potential benefit in cancer cachexia by attenuating the APPR.

This study investigates the production of pro-inflammatory cytokines by peripheral blood mononuclear cells isolated from patients with cancer, multiple organ failure (MOF) and healthy controls. The effect of such cytokines on the APPR is also investigated using an isolated human hepatocyte model. Finally, the effect of rhIL-4 and rhIL-2 on pro-inflammatory cytokine production by PBMC isolated from patients with MOF and cancer and controls is investigated and the effect of any such influence on the
ability of the PBMC supernatants to stimulate an acute phase protein response by isolated human hepatocytes.

MATERIALS AND METHODS

Isolation of peripheral blood mononuclear cells

Blood samples were taken from 6 healthy volunteers, 6 patients with multiple organ failure and 6 patients with gastrointestinal malignancy. Peripheral blood mononuclear cells were isolated as described in Chapter 2. Viable cells were estimated by trypan blue exclusion and were suspended at 1x10^6 /ml in supplemented RPMI. The cell suspension was plated at 200µl per well in 96 well flat-bottomed tissue culture plates (Falcon) in the presence or absence of recombinant human interleukin-4 (IL-4) (R&D Systems) at a final concentrations of 10 ng/ml. Mouse monoclonal anti-IL-4 (R&D Systems) was added where relevant at a final concentration of 1µg/ml. The control antibody was mouse hybridoma IgG (Sigma).

Lipopolysaccharide (LPS) from Escherichia coli 0127.B8 (Sigma) was added to test wells at a final concentration of 10µg/ml (Falcoher et al 1994) after 2 hr preliminary incubation at 37°C in 95% humidified air and 5% carbon dioxide. After a subsequent incubation of 18 hrs, supernatants were harvested and stored at -70°C for cytokine analysis and for incubation with hepatocytes.

TNF and IL-6 assays

Measurement of TNF and IL-6 in supernatants was performed by enzyme-linked immunosorbent assay (ELISA) as described in Chapter 2. For the TNF assay the limit of detection was 15pg/ml and for IL-6 75pg/ml. The interassay variations were 4% (Goldie et al 1995).

Isolation of human hepatocytes
Human hepatocytes were isolated as described in Chapter 2. Cells were plated at $3 \times 10^4$ per well in 96-well flat-bottomed tissue culture plates (Falcon), pre-coated with rat tail collagen (2µg/well) (Sigma) and incubated in 95% humidified air 5% CO$_2$ at 37°C. After 30 hours hepatocyte monolayers were washed with medium in preparation for the addition of supernatants from PBMC cultures.

**Acute phase protein production**

The supernatants from patient PBMC culture were diluted 1:10 in supplemented Williams medium and added to hepatocytes (200µl/well). Controls of medium only, recombinant IL-2, IL-4, IL-6, TNF and LPS were included. After 48 hours incubation, the supernatants were removed and stored at -70°C for measurement of acute phase protein production.

The production by hepatocytes of C-reactive protein (CRP), α1-antichymotrypsin (ACT), α1-acid glycoprotein (AGP), transferrin (TRF) and prealbumin (PREAL) were measured by sandwich ELISA (Chapter 2). The lower limit of sensitivity taking into account sample dilution was 120 pg/ml.

**Statistical analysis**

Results of cytokine production by PBMC are expressed as mean and standard error of the mean for each group. Results for acute phase protein production are expressed as percentage change compared with baseline (APP production in response to PBMC supernatants from controls which had not been exposed to LPS). APP production in response to control PBMC not exposed to LPS was chosen as a baseline since this represents the level of stimulation to production in a healthy subject and this represents therefore the state to which an effective therapeutic intervention would restore a subject from the disease groups. Statistical comparisons were performed with respect to baseline using Student’s 2-tailed paired t-test.
RESULTS

Patient characteristics

Patient characteristics of the three study groups are shown in Table 5.1. Controls were healthy volunteers who had no known active disease. The “Cancer” group were a heterogeneous group of patients with a variety of different gastrointestinal cancers. None of these patients had recently undergone surgery, endoscopic stenting or had received radiotherapy or cytotoxic chemotherapy. The group of patients with multiple organ failure were sampled as early as possible after the onset of their disease.

Table 5.1

Characteristics of patients in healthy control, cancer and multiple organ failure groups.

<table>
<thead>
<tr>
<th>Controls</th>
<th>Age</th>
<th>Sex</th>
<th>Time from onset of disease to sampling</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>F</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>F</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>F</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>52</td>
<td>M</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>F</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>M</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Age</th>
<th>Sex</th>
<th>Time from onset of disease to sampling</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72</td>
<td>M</td>
<td>2</td>
<td>Cholangiocarcinoma</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>F</td>
<td>3</td>
<td>Pancreas</td>
</tr>
<tr>
<td>3</td>
<td>69</td>
<td>F</td>
<td>1</td>
<td>Pancreas</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
<td>F</td>
<td>4</td>
<td>Colonic</td>
</tr>
<tr>
<td>5</td>
<td>53</td>
<td>F</td>
<td>5</td>
<td>Cholangiocarcinoma</td>
</tr>
<tr>
<td>6</td>
<td>67</td>
<td>M</td>
<td>23</td>
<td>Colonic</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MOF</th>
<th>Age</th>
<th>Sex</th>
<th>Time from onset of disease to sampling</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34</td>
<td>M</td>
<td>3</td>
<td>Acute pancreatitis</td>
</tr>
<tr>
<td>2</td>
<td>82</td>
<td>F</td>
<td>1</td>
<td>Mesenteric infarction</td>
</tr>
<tr>
<td>3</td>
<td>63</td>
<td>F</td>
<td>2</td>
<td>Cholangitis</td>
</tr>
<tr>
<td>4</td>
<td>57</td>
<td>F</td>
<td>7</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>M</td>
<td>3</td>
<td>Multiple trauma</td>
</tr>
<tr>
<td>6</td>
<td>22</td>
<td>F</td>
<td>5</td>
<td>Fulminant hepatic failure</td>
</tr>
</tbody>
</table>
IL-6 and TNF release by PBMC

Production of IL-6 and TNF was measured in PBMC culture supernatants following incubation in the presence and absence of LPS (10μg/ml) in each patient group. The effect of pre-incubation with rhIL-4 and rhIL-2 on IL-6 and TNF production by PBMC was also investigated.

The concentration of IL-6 in supernatants from unstimulated PBMC from healthy controls was 0.8 ng/ml ±0.5 (Table 5.2). This was not significantly different from production of IL-6 by unstimulated PBMC from cancer patients but was significantly higher than in patients with MOF (p<0.004). In the presence of lipopolysaccharide (LPS), significant increases in IL-6 production were observed in each group compared with unstimulated PBMC from controls.

The addition of rhIL-4 at 10ng/ml to unstimulated PBMC did not significantly reduce IL-6 release in any group. However, when rhIL-4 was added to PBMC which were subsequently stimulated with LPS significant reductions of IL-6 production were observed in all groups compared with LPS-stimulated IL-6 production in the absence of rhIL-4 (Table 5.2).

Table 5.2
Production of IL-6 (ng/ml) by peripheral blood mononuclear cells of healthy controls, patients with chronic inflammation (Cancer) and acute inflammation (multiple organ failure). Cells were isolated by density centrifugation on a Histopaque-1077 gradient and were plated at 2x10^5 cells per well. Cells were cultured in the presence and absence of lipopolysaccharide 10μg/ml with and without pre-incubation for 2 hours with IL-4 10ng/ml. Supernatants were harvested after 24 hours and assayed for IL-6 by ELISA.

<table>
<thead>
<tr>
<th></th>
<th>Spontaneous Nil</th>
<th>+IL-4</th>
<th>Lipopolysaccharide Nil</th>
<th>+IL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.8±0.5</td>
<td>0.3±0.3</td>
<td>7.4±0.7‡</td>
<td>3.3±2.1*</td>
</tr>
<tr>
<td>Cancer</td>
<td>0.6±0.6</td>
<td>0.2±0.3</td>
<td>8.0±0.6†</td>
<td>2.6±0.7*</td>
</tr>
<tr>
<td>MOF</td>
<td>0.1±0.2**</td>
<td>0.1±0.2</td>
<td>6.4±4.4*</td>
<td>1.6±3.1*</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.005, ‡ p<0.001, † p<0.0001 Student 2-tailed t-test versus baseline (Control PBMC cultured in the absence of LPS and IL-4)

Addition of rhIL-2 at either 1ng/ml or 10ng/ml had no effect on either the spontaneous or LPS-stimulated release of IL-6 by PBMC isolated from any group. (Table 5.3).
Table 5.3

Production of IL-6 (ng/ml) by peripheral blood mononuclear cells of healthy controls, patients with chronic inflammation (Cancer) and acute inflammation (multiple organ failure). Cells were isolated by density centrifugation on a Histopaque-1077 gradient and were plated at 2x10^5 cells per well. Cells were cultured in the presence and absence of lipopolysaccharide 10μg/ml with and without pre-incubation for 2 hours with IL-2 at a final concentration of either 1ng/ml or 10ng/ml. Supernatants were harvested after 24 hours and assayed for IL-6 by ELISA.

<table>
<thead>
<tr>
<th>Nil added</th>
<th>+IL-2 1ng/ml</th>
<th>+IL-2 10ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nil</td>
<td>LPS</td>
</tr>
<tr>
<td>Control</td>
<td>0.8±0.5</td>
<td>7.4±0.7</td>
</tr>
<tr>
<td>Cancer</td>
<td>0.6±0.3</td>
<td>8.0±0.6</td>
</tr>
<tr>
<td>MOF</td>
<td>0.1±0.2</td>
<td>6.4±1.8</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.005, †p<0.001, ‡p<0.0001 Student 2-tailed t-test versus baseline

The concentration of TNF in supernatants from unstimulated PBMC from healthy controls was 35.8 pg/ml ±10.9 (Table 5.4). There were no significant differences between TNF production by unstimulated PBMC from cancer patients and healthy controls. TNF production by PBMC of patients with MOF was significantly lower (P<0.04). The addition of LPS to PBMC significantly increased TNF release by PBMC from all groups (Table 5.4).

Pre-incubation with rhIL-4 decreased TNF release by PBMC in the cancer and MOF groups in the absence of LPS such that TNF concentration was significantly lower than that measured in healthy controls. In LPS-stimulated PBMC, TNF release was consistently reduced by rhIL-4 but remained greater than "nil" in all groups (Table 5.4).
Production of TNFα (pg/ml) by peripheral blood mononuclear cells of healthy controls, patients with chronic inflammation (Cancer) and acute inflammation (multiple organ failure). Cells were isolated by density centrifugation on a Histopaque-1077 gradient and were plated at 2x10^5 cells per well. Cells were cultured in the presence and absence of lipopolysaccharide 10μg/ml with and without pre-incubation for 2 hours with IL-4 10ng/ml. Supernatants were harvested after 24 hours and assayed for TNF by ELISA.

<table>
<thead>
<tr>
<th></th>
<th>Spontaneous</th>
<th>Lipopolysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nil</td>
<td>+IL-4</td>
</tr>
<tr>
<td>Control</td>
<td>35.8±10.9</td>
<td>22.9±24</td>
</tr>
<tr>
<td>Cancer</td>
<td>32.1±41.9</td>
<td>16.0±6.7*</td>
</tr>
<tr>
<td>MOF</td>
<td>13.8±12.6*</td>
<td>8.5±10.5*</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.005 Student 2-tailed t-test versus baseline (Control PBMC cultured in the absence of LPS and IL-4).
The addition of rhIL-2 at a concentration of either 1 or 10ng/ml had no effect on production of TNF by PBMC isolate from any group either in the presence or absence of LPS (Table 5.5).

Table 5.5

Production of TNFα (pg/ml) by peripheral blood mononuclear cells of healthy controls, patients with chronic inflammation (Cancer) and acute inflammation (multiple organ failure). Cells were isolated by density centrifugation on a Histopaque-1077 gradient and were plated at 2x10^5 cells per well. Cells were cultured in the presence and absence of lipopolysaccharide 10μg/ml with and without pre-incubation for 2 hours with IL-2 at a final concentration of either 1ng/ml or 10ng/ml. Supernatants were harvested after 24 hours and assayed for TNF by ELISA.

<table>
<thead>
<tr>
<th></th>
<th>Nil added</th>
<th>+IL-2 1ng/ml</th>
<th>+IL-2 10ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nil</td>
<td>LPS</td>
<td>Nil</td>
</tr>
<tr>
<td>Control</td>
<td>35.8±4.7</td>
<td>1701±296</td>
<td>60±18.4</td>
</tr>
<tr>
<td>Cancer</td>
<td>32±17</td>
<td>1305±457</td>
<td>12±4.5</td>
</tr>
<tr>
<td>MOF</td>
<td>14±5.2</td>
<td>1521±445</td>
<td>9.8±4.1</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.005, **p<0.001, †p<0.0001 Student 2-tailed t-test versus baseline

Table 5.6

Summary of the effects of IL-4 (10ng/ml) and IL-2 (1ng/ml and 10ng/ml) on production of IL-6 and TNF by PBMC isolated from controls and patients with cancer and multiple organ failure cultured in the absence (Nil) and Presence (LPS) of lipopolysaccharide 10μg/ml.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MOF</th>
<th>Cancer</th>
<th>Control</th>
<th>MOF</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>LP</td>
<td>0</td>
<td>LPS</td>
<td>0</td>
<td>LP</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>IL-4</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>IL-2 1ng/ml</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>IL-2 10ng/ml</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>10ng/ml</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
</tbody>
</table>
The addition of anti-IL-4 antibody at 10μg/ml suppressed the effect of rhIL-4 on both TNF and IL-6 production whereas the addition of an isotype control antibody had no effect (Table 5.7). The addition of anti-IL-2 antibody at 10μg/ml did not alter the effect of rhIL-2 at either concentration (Table 5.7).

Table 5.7

Production of IL-6 (ng/ml) and TNFα (pg/ml) by peripheral blood mononuclear cells of healthy controls. Cells were isolated by density centrifugation on a Histopaque-1077 gradient and were plated at 2x10^5 cells per well. Cells were cultured in the presence and absence of lipopolysaccharide 10μg/ml with and without pre-incubation for 2 hours with IL-4 or IL-2 at a final concentration of 10ng/ml in the presence and absence of either anti-IL-4 or anti-IL-2 antibody (10μg/ml) as appropriate or an isotype matched control antibody (10μg/ml). Supernatants were harvested after 24 hours and assayed for IL-6 and TNF by ELISA.

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th></th>
<th>TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nil</td>
<td>LPS</td>
<td>Nil</td>
</tr>
<tr>
<td>0</td>
<td>0.8±0.5</td>
<td>7.4±0.7</td>
<td>35.8±4.7</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.3±0.3</td>
<td>3.3±2.1</td>
<td>22.9±24</td>
</tr>
<tr>
<td>IL-4 + anti-IL-4</td>
<td>0.9±0.4</td>
<td>6.2±0.4</td>
<td>37.9±4.1</td>
</tr>
<tr>
<td>IL-4 + isotype control</td>
<td>0.4±0.1</td>
<td>3.4±1.3</td>
<td>21.6±3.4</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.33±0.4</td>
<td>7.3±0.8</td>
<td>71±21.9</td>
</tr>
<tr>
<td>IL-2 + anti-IL-2</td>
<td>1.36±0.7</td>
<td>8.0±1.1</td>
<td>56±7.8</td>
</tr>
<tr>
<td>IL-2 + isotype control</td>
<td>1.30±1.2</td>
<td>7.4±0.9</td>
<td>63±18.0</td>
</tr>
</tbody>
</table>

Hepatic acute phase protein synthesis

Supernatants from PBMC cultures were incubated with hepatocytes for 48h to examine their effect on both positive and negative acute phase protein synthesis. The results are presented as percentage change with respect to baseline values of acute phase proteins. The baseline was the concentration of APP measured in hepatocyte supernatants following the addition of unstimulated PBMC supernatants from controls.
This baseline was chosen to represent the state of healthy control subjects in the absence of endotoxin and the level to which successful immunomodulation with IL-4 or IL-2 would restore acute phase protein production to. The direct addition of rhIL-4 or rhIL-2 to hepatocytes alone had no effect on APP production.

Table 5.8

Effect of IL-4 10ng/ml and IL-2 10ng/ml on the production of acute phase proteins by isolated human hepatocytes.

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>IL-4 (10ng/ml)</th>
<th>IL-2 (10ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-1-antichymotrypsin</td>
<td>72.1±7.8</td>
<td>69.4±8.2</td>
<td>80.1±11.0</td>
</tr>
<tr>
<td>Alpha-1-acid glycoprotein</td>
<td>359.6±28.7</td>
<td>347±24.1</td>
<td>338±18.4</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>100.9±13.6</td>
<td>94±14.5</td>
<td>102.6±6.8</td>
</tr>
<tr>
<td>Transferrin</td>
<td>78.5±4.2</td>
<td>74.2±3.9</td>
<td>76.4±7.4</td>
</tr>
<tr>
<td>Prealbumin</td>
<td>76.5±8.7</td>
<td>82.0±9.1</td>
<td>84.7±5.2</td>
</tr>
</tbody>
</table>

Positive acute phase protein production

Alpha1-antichymotrypsin (ACT)

The effect of PBMC supernatants on ACT production by hepatocytes is shown in Figure 5.1a. The baseline value of ACT was 79.1 ±7.8 ng/ml. In this case production of ACT by hepatocytes in response to unstimulated PBMC from the MOF group was significantly greater than baseline (p<0.02). PBMC stimulation with LPS resulted in increased ACT production in response to PBMC supernatants in the MOF group. ACT production was reduced following addition of supernatants from PBMC pre-incubated with rhIL-4 such that there was no significant difference from baseline (MOF) or was significantly less than baseline (controls). A similar pattern of response was observed with respect to the effect of rhIL-4 on LPS-stimulated PBMC.
Production of the positive acute phase protein α1-antichymotrypsin (ACT) by isolated human hepatocytes. Hepatocyte cultures were incubated for 48h following the addition of PBMC supernatants from healthy volunteers (Controls), patients with gastrointestinal malignancy (cancer) and patients with multiple organ failure (MOF). PBMC supernatants were cultured for 24 hours in the presence and absence of lipopolysaccharide 10μg/ml with or without undergoing a 2 hour pre-incubation period with recombinant interleukin-4 10ng/ml. Supernatants were harvested from hepatocytes after 48 hours and assayed for the presence of acute phase proteins by ELISA. Results are expressed as a percentage of the baseline value of PBMC from healthy controls which had not been exposed to either IL-4 or lipopolysaccharide. The baseline values for ACT was 79.1±7.8 ng/ml. * denotes statistical significance p<0.05 vs Control 0 LPS, Student 2-tailed t test.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cancer</th>
<th>MOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 LPS</td>
<td>79.1±7.8</td>
<td>89.6±6.1</td>
<td>111±10.6</td>
</tr>
<tr>
<td>0 IL-4</td>
<td>91.7±10.5</td>
<td>96.5±7.1</td>
<td>155.2±13.1</td>
</tr>
<tr>
<td>LPS + IL-4</td>
<td>66.9±6.7</td>
<td>87.9±5.7</td>
<td>78.0±5.2</td>
</tr>
<tr>
<td>LPS+IL-4</td>
<td>67.8±6.5</td>
<td>86.3±5.4</td>
<td>89.4±5.4</td>
</tr>
</tbody>
</table>
Production of the positive acute phase protein α1-antichymotrypsin (ACT) by isolated human hepatocytes. Hepatocyte cultures were incubated for 48h following the addition of PBMC supernatants from healthy volunteers (Controls), patients with gastrointestinal malignancy (cancer) and patients with multiple organ failure (MOF). PBMC supernatants were cultured for 24 hours in the presence and absence of lipopolysaccharide 10μg/ml with or without undergoing a 2 hour pre-incubation period with recombinant interleukin-2 10ng/ml. Supernatants were harvested from hepatocytes after 48 hours and assayed for the presence of acute phase proteins by ELISA. Results are expressed as a percentage of the baseline value of PBMC from healthy controls which had not been exposed to either IL-2 or lipopolysaccharide. The baseline value for ACT was 79.1±7.8 ng/ml. * denotes statistical significance vs Control 0 LPS, p<0.05, † p<0.01 Student 2-tailed t test.

<table>
<thead>
<tr>
<th>Actual values of α1-antichymotrypsin ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>0 LPS</td>
</tr>
<tr>
<td>IL-2</td>
</tr>
<tr>
<td>LPS+IL-2</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

**Figure 5.2a.**
with attenuation of ACT production in response to supernatants from control and MOF groups such that there was no significant difference from baseline.

Addition of IL-2 to unstimulated PBMC supernatants had no effect on ACT production in controls and cancer patients but significantly reduced ACT production in response to supernatants from PBMC from patients with MOF (Figure 5.2a). Addition of rhIL-2 to LPS-stimulated PBMC reduced the ability of these supernatants to stimulate ACT production compared with non-rhIL-2-treated PBMC supernatants in all groups (Figure 5.2a).

Acid 1 glycoprotein (AGP)

The effects of PBMC supernatants on AGP production by hepatocytes are shown in Figure 5.1b. The baseline value of AGP was 359.6 ±28.7 ng/ml. Production of AGP by hepatocytes in response to the addition of unstimulated PBMC supernatants from the cancer and MOF groups was not different from baseline. There were significant increases in AGP production in the presence of LPS-stimulated PBMC supernatants compared with baseline in all groups.

AGP production in response to unstimulated PBMC pre-incubated with rhIL-4 (Figure 5.1b) was reduced in control (p<0.05) and MOF (p<0.05) groups but increased in the cancer group (p<0.05). In all groups the potential of LPS stimulated PBMC to elicit AGP was inhibited by pre-incubation with rhIL-4 but whilst reduction of AGP production to baseline levels was observed in controls, AGP remained significantly greater than baseline in the MOF (p<0.05) and cancer (p<0.05) groups.

AGP production in response to unstimulated PBMC pre-incubated with rhIL-2 (Figure 5.2b) was increased in cancer (p<0.01) but was unchanged in MOF and control groups. Similarly in the cancer group the potential of LPS stimulated PBMC to elicit AGP was increased (P<0.01) compared with MOF and control groups in which AGP production was not significantly different from baseline.
Addition of rhIL-2 to unstimulated PBMC supernatants had no effect on their ability to induce AGP production. When rhIL-2 was added to LPS-stimulate PBMC significant reduction in AGP production was observed in all three groups.

C-reactive protein (CRP)

The effects of PBMC supernatants on CRP production by hepatocytes is shown in Figure 5.1c. The baseline value of CRP was 100.9 ±13.6 ng/ml. Production of CRP by hepatocytes in response to the addition of unstimulated PBMC supernatants from the cancer and MOF groups were not different from baseline. CRP production was increased in response to supernatants from LPS-stimulated PBMC from control and cancer groups but not from the MOF group. The incubation of unstimulated PBMC with rhIL-4 reduced the potential of supernatants to stimulate CRP production by hepatocytes in all groups. Pre-incubation with rhIL-4 reduced the potential of LPS-stimulated PBMC to induce CRP production by hepatocytes in all groups, this reduction was not significantly different from baseline in the cancer group but was significantly lower than baseline in MOF and control groups.

Pre-incubation of unstimulated PBMC with rhIL-2 (Figure 5.2c) reduced the potential of supernatants to stimulate CRP production by hepatocytes in the MOF group (P<0.05) but had no effect on control and cancer groups. Pre-incubation with rhIL-2 reduced the potential of LPS-stimulated PBMC to induce CRP production by hepatocytes in the control and MOF groups (both p<0.05), but had no effect on CRP production in response to LPS-stimulated PBMC which had been isolated from the cancer group.
Production of the positive acute phase protein α1-acid glycoprotein (AGP) by isolated human hepatocytes. Hepatocyte cultures were incubated for 48h following the addition of PBMC supernatants from healthy volunteers (Controls), patients with gastrointestinal malignancy (cancer) and patients with multiple organ failure (MOF). PBMC supernatants were cultured for 24 hours in the presence and absence of lipopolysaccharide 10μg/ml with or without undergoing a 2 hour pre-incubation period with recombinant interleukin-4 10ng/ml. Supernatants were harvested from hepatocytes after 48 hours and assayed for the presence of acute phase proteins by ELISA. Results are expressed as a percentage of the baseline value of PBMC from healthy controls which had not been exposed to either IL-4 or lipopolysaccharide. The baseline value for AGP was 359.6±28.7 ng/ml. * denotes statistical significance p<0.05 Student 2-tailed t test.

<table>
<thead>
<tr>
<th>Actual values of α1-acid glycoprotein ng/ml</th>
<th>Control</th>
<th>Cancer</th>
<th>MOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>359.6±28.7</td>
<td>421.1±56.6</td>
<td>296.5±18</td>
</tr>
<tr>
<td>LPS</td>
<td>458.1±46.9</td>
<td>672.3±56.9</td>
<td>635.5±79.6</td>
</tr>
<tr>
<td>IL-4</td>
<td>278.9±35.3</td>
<td>437.8±28.3</td>
<td>213.2±17.6</td>
</tr>
<tr>
<td>LPS+IL-4</td>
<td>363.5±49.2</td>
<td>531.3±38.1</td>
<td>443.7±43.9</td>
</tr>
</tbody>
</table>
Production of the positive acute phase protein α1-acid glycoprotein (AGP) by isolated human hepatocytes. Hepatocyte cultures were incubated for 48h following the addition of PBMC supernatants from healthy volunteers (Controls), patients with gastrointestinal malignancy (cancer) and patients with multiple organ failure (MOF). PBMC supernatants were cultured for 24 hours in the presence and absence of lipopolysaccharide 10μg/ml with or without undergoing a 2 hour pre-incubation period with recombinant interleukin-2 10ng/ml. Supernatants were harvested from hepatocytes after 48 hours and assayed for the presence of acute phase proteins by ELISA. Results are expressed as a percentage of the baseline value of PBMC from healthy controls which had not been exposed to either IL-2 or lipopolysaccharide. The baseline value for AGP was 359.6±28.7 ng/ml. * denotes statistical significance p<0.05, † p<0.01, ‡‡ p<0.001 Student 2-tailed t test.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cancer</th>
<th>MOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 LPS</td>
<td>359.6±28.7</td>
<td>421.1±56.6</td>
<td>296.5±18</td>
</tr>
<tr>
<td>IL-2</td>
<td>458.1±46.9</td>
<td>672.3±56.9</td>
<td>635.5±79.6</td>
</tr>
<tr>
<td>LPS+IL-2</td>
<td>335.4±41.5</td>
<td>475.1±34.1</td>
<td>334.8±28.1</td>
</tr>
<tr>
<td></td>
<td>350.6±42.2</td>
<td>542.9±38.0</td>
<td>352.6±24.6</td>
</tr>
</tbody>
</table>
Production of the positive acute phase protein C-reactive protein (CRP) by isolated human hepatocytes. Hepatocyte cultures were incubated for 48h following the addition of PBMC supernatants from healthy volunteers (Controls), patients with gastrointestinal malignancy (cancer) and patients with multiple organ failure (MOF). PBMC supernatants were cultured for 24 hours in the presence and absence of lipopolysaccharide 10μg/ml with or without undergoing a 2 hour pre-incubation period with recombinant interleukin-4 10ng/ml. Supernatants were harvested from hepatocytes after 48 hours and assayed for the presence of acute phase proteins by ELISA. Results are expressed as a percentage of the baseline value of PBMC from healthy controls which had not been exposed to either IL-4 or lipopolysaccharide. The baseline value for CRP was 100.9±13.6 ng/ml. * denotes statistical significance p<0.05 Student 2-tailed t test.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cancer</th>
<th>MOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.9±13.6</td>
<td>114.2±12.4</td>
<td>86.4±8.6</td>
</tr>
<tr>
<td>LPS</td>
<td>146.1±33.5</td>
<td>140.0±24.1</td>
<td>85.3±6.2</td>
</tr>
<tr>
<td>IL-4</td>
<td>68.8±8.3</td>
<td>85.2±9.2</td>
<td>51.4±5.0</td>
</tr>
<tr>
<td>LPS+IL-4</td>
<td>70.3±8.0</td>
<td>111.2±16.4</td>
<td>68.0±7.2</td>
</tr>
</tbody>
</table>

* denotes statistical significance p<0.05 Student 2-tailed t test.
Production of the positive acute phase protein C-reactive protein (CRP) by isolated human hepatocytes. Hepatocyte cultures were incubated for 48h following the addition of PBMC supernatants from healthy volunteers (Controls), patients with gastrointestinal malignancy (cancer) and patients with multiple organ failure (MOF). PBMC supernatants were cultured for 24 hours in the presence and absence of lipopolysaccharide 10µg/ml with or without undergoing a 2 hour pre-incubation period with recombinant interleukin-2 10ng/ml. Supernatants were harvested from hepatocytes after 48 hours and assayed for the presence of acute phase proteins by ELISA. Results are expressed as a percentage of the baseline value of PBMC from healthy controls which had not been exposed to either IL-2 or lipopolysaccharide. The baseline value for CRP was 100.9±13.6 ng/ml. * denotes statistical significance p<0.05 Student 2-tailed t test.

<table>
<thead>
<tr>
<th>Actual values of C-reactive protein ng/ml</th>
<th>Control</th>
<th>Cancer</th>
<th>MOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.9±13.6</td>
<td>114.2±12.4</td>
<td>86.4±8.6</td>
</tr>
<tr>
<td>LPS</td>
<td>146.1±33.5</td>
<td>140.0±24.1</td>
<td>85.3±6.2</td>
</tr>
<tr>
<td>IL-2</td>
<td>70.5±10.9</td>
<td>123.3±16.5</td>
<td>56.3±4.0</td>
</tr>
<tr>
<td>LPS+IL-2</td>
<td>61.1±6.1</td>
<td>135.3±29.2</td>
<td>48.0±4.2</td>
</tr>
</tbody>
</table>
Negative acute phase protein production

Transferrin (TRF)

The effects of PBMC supernatants on TRF production by hepatocytes is shown in Figure 5.1d. The baseline value of TRF was 78.5 ± 4.2 ng/ml. TRF release in response to PBMC supernatants from cancer patients was significantly greater than baseline (p<0.05). TRF production in response to LPS stimulated PBMC supernatants was unchanged in the control group, significantly greater than baseline in the cancer group and was significantly lower than baseline in the MOF group. Following pre-incubation of PBMC with rhIL-4 and in the absence of LPS, TRF production by hepatocytes was unchanged in control and MOF groups but remained elevated in the cancer group. In the presence of LPS pre-incubation with rhIL-4 had no effect on TRF production compared with LPS stimulated PBMC alone.

Following pre-incubation of PBMC with rhIL-2 and in the absence of LPS, TRF production by hepatocytes was unchanged in the control group, was significantly reduced in the MOF group (p<0.05), but remained elevated in the cancer group (Figure 5.2d). In the presence of LPS pre-incubation with rhIL-2 had no effect on TRF production compared with LPS stimulated PBMC alone.
Figure 5.1d.

Production of the negative acute phase protein transferrin (TRF) by isolated human hepatocytes. Hepatocyte cultures were incubated for 48h following the addition of PBMC supernatants from healthy volunteers (Controls), patients with gastrointestinal malignancy (cancer) and patients with multiple organ failure (MOF). PBMC supernatants were cultured for 24 hours in the presence and absence of lipopolysaccharide 10\mu g/ml with or without undergoing a 2 hour pre-incubation period with recombinant interleukin-4 10ng/ml. Supernatants were harvested from hepatocytes after 48 hours and assayed for the presence of acute phase proteins by ELISA. Results are expressed as a percentage of the baseline value of PBMC from healthy controls which had not been exposed to either IL-4 or lipopolysaccharide. The baseline value for TRF was 78.5±4.2 ng/ml. * denotes statistical significance p<0.05 Student 2-tailed t test.

![Graph showing % change vs baseline for controls, cancer, and MOF under spontaneous and lipopolysaccharide conditions with actual transferrin values listed in table.]
Production of the negative acute phase protein transferrin (TRF) by isolated human hepatocytes. Hepatocyte cultures were incubated for 48h following the addition of PBMC supernatants from healthy volunteers (Controls), patients with gastrointestinal malignancy (cancer) and patients with multiple organ failure (MOF). PBMC supernatants were cultured for 24 hours in the presence and absence of lipopolysaccharide 10µg/ml with or without undergoing a 2 hour pre-incubation period with recombinant interleukin-2 10ng/ml. Supernatants were harvested from hepatocytes after 48 hours and assayed for the presence of acute phase proteins by ELISA. Results are expressed as a percentage of the baseline value of PBMC from healthy controls which had not been exposed to either IL-2 or lipopolysaccharide. The baseline value for TRF was 78.5±4.2 ng/ml. * denotes statistical significance p<0.05 Student 2-tailed t test.

<table>
<thead>
<tr>
<th>Actual values of transferrin</th>
<th>Control</th>
<th>Cancer</th>
<th>MOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>78.5±4.2</td>
<td>119.4±23.3</td>
<td>73.8±4.5</td>
</tr>
<tr>
<td>LPS</td>
<td>73.2±6.5</td>
<td>130.5±13.5</td>
<td>50.5±3.7</td>
</tr>
<tr>
<td>IL-2</td>
<td>73.1±6.8</td>
<td>123.3±19.3</td>
<td>59.3±7.1</td>
</tr>
<tr>
<td>LPS+IL-2</td>
<td>77.1±8.1</td>
<td>132.0±25.7</td>
<td>70.8±9.6</td>
</tr>
</tbody>
</table>
Prealbumin (PREAL)

The effects of PBMC supernatants on PREAL production by hepatocytes is shown in Figure 5.1e. The baseline value of PREAL was 76.5 ±8.7 ng/ml. Production of PREAL in response to unstimulated PBMC supernatants was significantly lower than baseline in cancer patients (p<0.05). Also in this group, stimulation of PBMC with LPS resulted in further reduction of PREAL production. Pre-incubation of PBMC with rhIL-4 had no effect on the potential of unstimulated cells to induce prealbumin production. PREAL production was significantly lower than baseline in response to supernatants from LPS-stimulated PBMC which had been pre-incubated with rhIL-4 in all groups. Pre-incubation of PBMC with rhIL-2 (Figure 5.2e) had no effect on the potential of unstimulated cells isolated from cancer and MOF groups to induce PREAL production but significantly reduced the potential of control PBMC to induce PREAL production (p<0.05). Pre-incubation with rhIL-2 had no effect on the potential of LPS-stimulated PBMC to influence PREAL production.
Production of the negative acute phase protein prealbumin (PREAL) by isolated human hepatocytes. Hepatocyte cultures were incubated for 48h following the addition of PBMC supernatants from healthy volunteers (Controls), patients with gastrointestinal malignancy (cancer) and patients with multiple organ failure (MOF). PBMC supernatants were cultured for 24 hours in the presence and absence of lipopolysaccharide 10ug/ml with or without undergoing a 2 hour pre-incubation period with recombinant interleukin-4 10ng/ml. Supernatants were harvested from hepatocytes after 48 hours and assayed for the presence of acute phase proteins by ELISA. Results are expressed as a percentage of the baseline value of PBMC from healthy controls which had not been exposed to either IL-4 or lipopolysaccharide. The baseline value for PREAL was 76.5±8.7 ng/ml. * denotes statistical significance p<0.05 Student 2-tailed t test.

![Graph showing % change vs baseline](image)

<table>
<thead>
<tr>
<th>Actual values of prealbumin ng/ml</th>
<th>Control</th>
<th>Cancer</th>
<th>MOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>76.5±8.7</td>
<td>60.1±6.8</td>
<td>87.8±5.9</td>
</tr>
<tr>
<td>LPS</td>
<td>68.3±8.6</td>
<td>45.4±4.6</td>
<td>84.4±13.3</td>
</tr>
<tr>
<td>IL-4</td>
<td>70.5±6.2</td>
<td>58.1±7.9</td>
<td>72.3±4.8</td>
</tr>
<tr>
<td>LPS+IL-4</td>
<td>52.9±6.3</td>
<td>43.6±3.7</td>
<td>54.0±4.8</td>
</tr>
</tbody>
</table>
Production of the negative acute phase protein prealbumin (PREAL) by isolated human hepatocytes. Hepatocyte cultures were incubated for 48h following the addition of PBMC supernatants from healthy volunteers (Controls), patients with gastrointestinal malignancy (cancer) and patients with multiple organ failure (MOF). PBMC supernatants were cultured for 24 hours in the presence and absence of lipopolysaccharide 10μg/ml with or without undergoing a 2 hour pre-incubation period with recombinant interleukin-4 10ng/ml. Supernatants were harvested from hepatocytes after 48 hours and assayed for the presence of acute phase proteins by ELISA. Results are expressed as a percentage of the baseline value of PBMC from healthy controls which had not been exposed to either IL-4 or lipopolysaccharide. The baseline value for PREAL was 76.5±8.7 ng/ml. * denotes statistical significance p<0.05, † p<0.01 Student 2-tailed t test.

<table>
<thead>
<tr>
<th>Actual values of prealbumin ng/ml</th>
<th>Control</th>
<th>Cancer</th>
<th>MOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>76.5±8.7</td>
<td>60.1±6.8</td>
<td>87.8±5.9</td>
</tr>
<tr>
<td>LPS</td>
<td>68.3±8.6</td>
<td>45.4±4.6</td>
<td>84.4±13.3</td>
</tr>
<tr>
<td>IL-2</td>
<td>56.6±5.8</td>
<td>61.9±5.6</td>
<td>83.0±13.5</td>
</tr>
<tr>
<td>LPS+IL-2</td>
<td>74.8±10.1</td>
<td>92.6±19.4</td>
<td>61.9±6.3</td>
</tr>
</tbody>
</table>
Summary of the effects of incubation with IL-4 10ng/ml and IL-2 10ng/ml with PBMC isolated from controls and patients with cancer and multiple organ failure on the ability of PBMC supernatants to stimulate acute phase protein production by isolated human hepatocytes.

<table>
<thead>
<tr>
<th></th>
<th>ACT</th>
<th>AGP</th>
<th>CRP</th>
<th>TRF</th>
<th>PREAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>nil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lps</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>il-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>il-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+lps</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>il-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>il-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+lps</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

In this study, LPS-induced production of both IL-6 and TNF from PBMC was attenuated by the presence of IL-4. These observations are in agreement with published work on the effect of IL-4 on pro-inflammatory cytokine release by PBMC. Lee and co-workers demonstrated that the effect of IL-4 on PBMC pro-inflammatory cytokine release was time dependent and was maximally effective if introduced before the LPS stimulus. Other workers, however, have demonstrated almost complete abolition of PBMC cytokine production following simultaneous addition of LPS and IL-4 (te Velde et al, 1990).

Cytokine production by unstimulated PBMC from MOF patients was significantly lower than baseline which may represent deteriorating function of these cells secondary to previous in vivo activation. It is well recognised that seriously ill patients in an intensive care unit setting develop immune dysfunction and this specific phenomenon has been previously described in PBMC isolated from patients with sepsis (Ertel et al, 1995). Pre-incubation of IL-4 with PBMC from both MOF and cancer patients resulted in LPS-induced down regulation of LPS-induced pro-inflammatory cytokine production. These results demonstrate that PBMC from patients with acute and chronic disease states are not refractory to down-regulation by IL-4. However, in the case of LPS-stimulated PBMC, cytokine concentrations were not reduced to baseline in any group, indeed within the restrictions of this experimental model there were no major differences in response between either of the patient groups or controls.

In controls production of all the positive acute phase proteins from hepatocytes was increased in the presence of supernatants from PBMC exposed to LPS. Recombinant IL-6 has been shown to suppress synthesis of the negative acute phase proteins (Heinrich). However, in the present study production of the negative acute phase proteins TRF and PRE was unaffected by the addition of the supernatants from
LPS-stimulated cells. This in vitro observation would agree with our in vivo observations (Falconer et al., 1994) using stable isotope technology which demonstrated that the albumin synthetic rate was not reduced in patients with a continuing acute phase response.

Using PBMC supernatants from healthy controls complex differential regulation of the positive and negative acute phase proteins was observed when supernatants from unstimulated or LPS-stimulated PBMC incubated in the absence or presence of IL-4, were added to hepatocyte cultures. The inclusion of IL-4 in the PBMC cultures not exposed to LPS reduced the subsequent production by hepatocytes of the positive acute phase proteins but did not affect the production of the negative acute phase proteins TRF and PREAL. However, in hepatocytes exposed to supernatants from LPS-stimulated PBMC incubated in the presence of IL-4, the production of ACT and AGP was reduced to baseline levels whilst the production of CRP was reduced below this baseline. Similarly, production of the negative acute phase protein PREAL was reduced when hepatocytes were exposed supernatants from PBMC exposed to LPS in the presence of IL-4. It has previously been reported that IL-4 has a direct effect suppressing production of acute phase proteins (Loyer et al, 1993). In that study hepatocytes were cultured in the presence of hydrocortisone and insulin. It has previously been demonstrated that such counter regulatory hormones can exert a profound modifying effect on hepatocyte acute phase protein production both in isolation and in combination (O'Riordan et al, 1995). In the present study hepatocytes were cultured in the absence of any added hormones and the medium used was supplemented with foetal calf serum which had been heat inactivated. IL-4 alone had no effect on the production of the various acute phase proteins. Furthermore, administration of IL-4 to cancer patients resulted in evidence of hepatotoxicity in two clinical trials and it is possible that the effects of IL-4 on hepatocyte acute phase protein production and mRNA expression in that study was in some way related to a direct toxic effect (Gilleece et al 1992, Margolin et al, 1994). Whether IL-4 had a toxic effect
in the present study is uncertain however the direct addition of IL-4 to hepatocytes did not significantly alter acute phase protein production compared with control supernatants (Table 5.7).

Turning to the effects of IL-4 in MOF and Cancer, (Table 5.3) it is clear that different patterns of production of a number of hepatic export proteins is induced by the supernatants of PBMC from different patient groups. This casts some doubt on the use of CRP alone as a clinical indicator of the presence of an acute phase response. It is also clear that there is a differential effect of IL-4 on the production of a number of both normal export proteins and the positive acute phase proteins in the presence of supernatants from different patient groups.

In this study the effect of IL-2 on pro-inflammatory cytokine production and the ability of PBMC to elicit an APPR was investigated. It has been shown that IL-2 can exert different biological effects dependent on its effective concentration. At low concentrations IL-2 is thought to act principally by stimulating T-lymphocyte differentiation, lymphocyte tumoricidal activity and activation of NK and LAK cells. At higher concentrations IL-2 has been reported to stimulate IL-6 and TNF production and to cause histamine release from basophils. Trials of IL-2 in cancer have been associated with considerable toxicity particularly when high doses of IL-2 are used and it is believed that this may in part represent the dose dependent activation of pro-inflammatory cytokine release Deehan et al, 1994). The rationale for using IL-2 in the context of this study was that IL-2 is known to induce the production of the regulatory or type 2 cytokines IL-4 and IL-10 by lymphocytes. Since PBMC comprise a mixed population of monocytes and lymphocytes it was proposed that the addition of exogenous IL-2 to PBMC might stimulate IL-4 and IL-10 production and that this might result in suppression of monocyte pro-inflammatory cytokine production. At the time that these experiments were conducted there were no commercially available pairs of antibodies with which to develop an ELISA to measure IL-10. In addition
preliminary experiments by another member of the laboratory staff (Dr J.A. Ross) indicated that IL-4 could only be measured in supernatants of lymphocyte containing cultures following maximal stimulation and that the accuracy of the then available assay did not justify the considerable expense of attempting to measure IL-4 in the experimental model described here. The inability to measure IL-4 and IL-10 is clearly a major limitation of the present study and confirmatory evidence of the effect of IL-2 on IL-4 and IL-10 release would have been helpful in assessing the effectiveness of this experimental model. In the present study IL-2 was used at a dose of 1ng/ml and 10ng/ml. Addition of rhIL-2 at either a low dose or a high dose had no effect on TNF or IL-6 production by PBMC from any group. IL-2 has been previously described as being capable of inducing IL-6 production by monocytes however no such effect was observed in the present study.

APPR production in response to supernatants from unstimulated PBMC which had been incubated with IL-2 was unchanged in the case of AGP, and TRF and only reduced in MOF in the case of ACT and CRP. Incubation with IL-2 reduced the stimulation of PREAL in response to unstimulated PBMC supernatants from controls but had no effect in the other two groups. Pre-incubation of IL-2 with LPS stimulated PBMC significantly reduced production of ACT and AGP in all groups and of CRP in controls and MOF patients. This was an unexpected observation because IL-2 did not have any direct effect on hepatocyte production and also did not significantly change the concentrations of IL-6 or TNF in PBMC supernatants. It must be assumed that IL-2 has altered the concentration of some other factor regulating hepatocyte acute phase production. The nature of this factor is uncertain. Turning to the effects of IL-2 on the ability of LPS-stimulated PBMC to elicit negative acute phase protein production; production of TRF was increased in MOF patients but not in the other two groups. In the case of PREAL production was increased in cancer patients but unaffected in the other groups.
The different patterns of acute phase protein production induced by supernatants from PBMC of surgical patients with either acute or chronic illness and their different responses to IL-4 and IL-2 suggests the presence of multiple regulatory factors which are released from the PBMC of patients. The understanding of the results observed including some unexpected observations might have been easier had serum acute phase protein concentrations been measured. Although IL-6 is thought to be the primary regulator of the acute phase response in vivo other cytokines, including IL-1, TNF, TGFβ, IL-11, ciliary neurotrophic factor, oncostatin M and LIF, can also regulate many of the acute phase genes. The presence of various combinations of cytokines in the PBMC supernatants from different patient groups may produce differential effects on subsets of acute phase proteins. These findings suggest that more detailed studies in vitro of regulatory mechanisms will be required before clinical studies of biomodulation would be justified.

In vivo the hepatocyte does not function in isolation but is influenced by cytokine production from adjacent Kupffer cells, monocytes and also endothelial cells. de Beaux et al., 1995 cultured endothelial cells in vitro and have investigated the modulation of endothelial pro-inflammatory (IL-6 and IL-8) cytokine production by IL-4 and IL-10 (Bogdan et al, 1993). They have demonstrated that regulatory cytokines such as IL-4 and IL-10 increase the production of pro-inflammatory cytokines from endothelial cells. This is in contrast to the published data on the effect of IL-4 and IL-10 in down-regulating the production of IL-6 from cells of the monocyte-macrophage series. This work emphasises the pleiotropy of cytokines and the complex regulatory mechanisms which exist and indicates the difficulties in devising in vivo intervention strategies with such cytokines.
Chapter 6.

ENDOGENOUS INTERLEUKIN-8 AND INTERLEUKIN-6 PRODUCTION BY HUMAN PANCREATIC CANCER CELLS AND THEIR EFFECT ON THE HEPATIC ACUTE PHASE RESPONSE.
INTRODUCTION

Patients with pancreatic cancer have been shown to have both an increased resting energy expenditure and a chronically elevated hepatic acute phase protein response (Falconer et al, 1994). The importance of an elevated acute phase protein response in cancer cachexia has been reinforced by the finding that an elevated serum C-reactive protein concentration at the time of diagnosis is the strongest independent predictor of poor prognosis, exceeding the predictive value of all conventional criteria including stage of disease (Falconer et al, 1995). The alterations in metabolism accounting for the reprioritisation of hepatic protein synthesis in favour of acute phase protein production, are thought to be mediated by IL-6, IL-1 and TNF (Heinrich et al, 1990), and it has been suggested that these cytokines may account in part for many of the metabolic abnormalities and consequent tissue wasting observed in the cancer host (Fearon, 1992). Despite compelling data demonstrating the potent activity of IL-6, IL-1 and TNF in stimulating acute phase protein production in vitro (Majello et al, 1990; Morrone et al, 1988; Jablons et al, 1989), in animal models (Oliff et al, 1987; Stovroff et al, 1989) and in humans (Castell et al, 1990) these factors are often not detectable in the sera of cancer patients with an acute phase response (Falconer et al, 1994). The initiating stimulus for acute phase protein production in pancreatic cancer therefore remains obscure.

IL-8 is a pro-inflammatory cytokine whose principal role in infection and inflammation appears to be the recruitment and activation of circulating and tissue neutrophils and lymphocytes to the site of tissue damage. It has been demonstrated that IL-8 is produced by a wide variety of cell types in vitro including vascular and lung endothelium (Luscinskas et al, 1990; Mulligan et al, ), monocytes (Becker et al, 1992; Friedland et al, 1992; Gusella et al, 1993), eosinophils (Braun et al, 1993), kidney mesangial cells (Abbot et al, 1991), astrocytes (Aloisi et al, 1992) and keratinocytes (Kristensen et al, 1991). Studies have demonstrated that IL-1β and TNF-α induce transcriptional activation of the IL-8 gene and synthesis of IL-8 protein (Abbot et al, 1991). In addition to its pro-inflammatory effects it has recently been demonstrated that
IL-8 is a potent angiogenic factor and that it might therefore have a role in tumor proliferation and implantation (Koch et al, 1992).

This study investigates the role of tumor-derived cytokines in the aetiology of the acute phase protein response. It describes the production of IL-8 and IL-6 by human pancreatic cell lines \textit{in vitro} and investigates the effect of these cytokines on the hepatic acute phase protein response \textit{in vitro} using both isolated human hepatocytes and cultures of a human hepatoma cell line (HepG2).
MATERIALS AND METHODS

Pancreatic cancer cell lines.

The adherent human pancreatic cancer cell lines MIAPaCa2, CFPAC and PANC 1 were obtained from the European Collection of Animal Cell Cultures (ECACC, PHLS Centre for Applied Microbiology and Research, Porton Down, UK) were plated out at a concentration of $5 \times 10^3$ in 96 well tissue culture plates (Costar, High Wycombe, UK) in 200μl of medium per well. Cells were incubated in Dulbecco's modification of Eagle's medium (DMEM) (GIBCO-BRL, Inchinan, UK) supplemented with 5% heat-inactivated foetal calf serum (GIBCO-BRL, Inchinan, UK), penicillin (50IU/ml) and streptomycin (50μg/ml) (ICN Biomedicals, Irvine, UK) and 2mM L-glutamine (ICN Biomedicals, Irvine, UK) at 37°C in 95% humidified air and 5% CO₂. Cells were allowed to adhere for 24 hours and then washed using fresh medium prior to incubation in medium as detailed above with the addition of any further supplements as detailed below.

Endogenous cytokine production by human pancreatic cancer cells.

Pancreatic cancer cells were cultured in batches as described above. In timed experiments, cells were counted at 0, 24, 48, 72, 96, 120, 144 and 168 hours following trypsinisation and staining with trypan blue, which also permitted evaluation of cell viability. Supernatants were removed and stored at -70°C until assay.

Evaluation of the effect of IL-1β, TNFα and IL-4 on endogenous cytokine production by pancreatic cancer cell lines.

Cells from each cell line were plated in 96-well plates as described above. Recombinant IL-1β and TNFα (R&D Systems, Abingdon, UK) were added at final concentrations of 100, 10, 1, 0.1, 0.01 and 0 ng/ml, either singly or in combination. Recombinant human IL-4 (Genzyme, West Malling, UK) was used in a fixed final
concentration of 1ng/ml in all experiments. Following addition of cytokines the cells were incubated for 24 hours prior to analysis of cytokine production.

*mRNA Extraction and reverse transcription*

The reverse transcription-PCR method was used to detect IL-8 mRNA in pancreatic cancer cell lines and was performed by Mr P.B.S. Lai, Mrs J.P. Maingay and Dr J.A. Ross. Poly(A)+mRNA was purified from MIA PaCa-2 cells using a fast track mRNA purification kit (Invitrogen, San Diego, USA). One µg of the poly(A)+mRNA was then reverse transcribed using a reverse transcription kit (Promega, Madison, USA) according to the manufacturers instructions. A 20µl reaction consisted of 5mM MgCl2, 1mM deoxynucleotides, 0.5µg oligo (dT) primer, 2µl 10x reverse transcription buffer (500mM KCL, 100mM Tris-HCL, 1% Triton X-100), 20 U RNAsin ribonuclease inhibitor and 15 U AMV reverse transcriptase. The reverse transcription was iactivated by heating the reaction mixture at 99°C for 5 minutes followed by a 5 minute incubation at 0°C. The reaction mixture containing cDNAs was stored at -20°C.

*Polymerase chain reaction (PCR)*

After reverse transcription to cDNA, amplification was carried out by PCR. The sense and anti-sense IL-8 primers were (‘5->’3) ATGACTTCCAAGCTGGCGTGGCT and TCTCAGCCCTCTTCAAAAAACTTCTC respectively (Oswel, Southampton, UK). The predominant cDNA amplification product was predicted to be 289 base pair (bp) for IL-8 and 983 bp and 1000 bp for glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) and bcl-2 respectively which were used as positive controls. A typical 10µl PCR reaction mixture consisted of 1.25mM MgCl2, 0.2mM dNTP mixture (Promega), 1 µl cDNA RT sample, 1µM 3’ and 5’ primer, 1µl 10 x PCR buffer (500mM KCl, 100mM Tris-HCL, 1% Triton X-100) and 0.5 U Taq polymerase (Promega). An overlay of 15µl mineral oil (Sigma, Poole, UK)
was added to prevent evaporation during high temperature incubations. The PCR profile was 95°C for 1 minute, 65°C for 1 minute and 72°C for 2 minutes for 40 cycles. After PCR, 5μl aliquots of PCR reaction mixtures were analysed on 1% TAE agarose gel with 0.5μg/ml ethidium bromide (Sigma, Poole, UK) and photographed using Polaroid Type 665 film.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS PAGE analysis was performed on supernatants from pancreatic cancer cell lines (Schagger & von Jagow, 1987). The separating cell was composed of 16.5% T and 6% C acrylamide/bisacrylamide polymer containing 10.8 g urea and gel buffer (3M Tris, 0.1M tricine and 0.1% sodium dodecyl sulphate (SDS)) to which 150μl ammonium persulphate and 15μl TEMED /30ml was added. After casting, the gel was covered with a thin layer of isobutanol to prevent dessication while setting. After 40 minutes the stacking gel (4%T and 3% C) was laid over the separating gel using a comb. The gel and plates were transferred to the cell (Biorad Protean ix, Biorad, UK). The cathode buffer was 0.1M Tris, 0.1M tricine and 0.1% SDS corrected to pH 8.25 and the anode buffer was 0.2M Tris at pH 8.9. Samples were added to sample buffer (4% SDS, 12% glycerol, 50mM Tris, 2% mercaptoethanol and 0.01% bromophenol blue) in a ration of 1:1. Samples were heated at 40°C for 40 minutes and were then underlaid using fine glass pipettes onto the gel lanes. A series of low and high rainbow molecular weight markers were run in each experiment. Positive controls were medium containing 1μg/ml IL-8 and IL-8 at the same concentration in carrier and the negative control was medium only. Electrophoresis was started at 30V constant and then increased to 65V to give a speed of the gel front of 0.8cm per hour.

Western blot analysis

Following SDS-PAGE gels were transferred onto nitrocellulose membranes using a current of 0.8mA / cm². Following transfer the molecular weight marker lanes were removed and the nitrocellulose membranes were incubated in a solution of 1%
Marvel to block the membrane. The membranes were then incubated with anti-human IL-8 monoclonal antibody at a concentration of 5µg/ml in 1% Marvel and Tween 20 solution. The membranes were then washed and incubated with goat anti-human antibody. Following further washing the membrane was incubated with a biotinylated anti-goat antibody (Dako). The membrane was then developed using ABC Streptavidin complex diluted 1:100 in PBS containing 3% Tween and DAB.

**Isolation of human hepatocytes**

Hepatocytes were isolated and cultured as described in Chapter 2. Recombinant carrier-free human IL-8 or IL-6 (R&D Systems, Abingdon, UK) was used in all experiments at the following final concentrations: 0.01, 0.1, 1.0, 10 or 100 ng/ml. Following the addition of medium containing cytokines, hepatocytes were incubated for 24 hours at which time supernatants were harvested for measurement of acute phase proteins.

**Culture of human hepatoma cell line Hep G2**

The adherent human hepatoma cell line Hep G2 was obtained from the European Collection of Animal Cell Cultures (ECACC, PHLS Centre for Applied Microbiology and Research, Porton Down, UK). Cells were plated out at a concentration of 3x 10⁴ in 96 well tissue culture plates (Costar, High Wycombe, UK) in 200µl of medium per well. Cells were incubated in Dulbecco's modification of Eagle's medium (DMEM) (GIBCO-BRL, Inchinan, UK) supplemented with 5% heat-inactivated foetal calf serum (GIBCO-BRL, Inchinan, UK), penicillin (50IU/ml) and streptomycin (50µg/ml) (ICN Biomedicals, Irvine, UK) and 2mM L-glutamine (ICN Biomedicals, Irvine, UK) at 37°C in 95% humidified air and 5% CO₂. Cells were allowed to adhere for 24 hours and then washed using fresh medium prior to incubation in medium as detailed above with the addition of any further supplements as detailed below.
Measurement of C-reactive protein, IL-6 and IL-8

Sandwich enzyme-linked immunosorbent assays (ELISAs) were employed for the measurement of C-reactive protein (CRP), IL-6 and IL-8 as described in Chapter 2. The limits of sensitivity of the assays, taking into consideration the sample dilutions, were 120 pg/ml for CRP, 40 pg/ml for IL-6 and 190 pg/ml for IL-8.

Statistics

Analysis of significance between variables was performed using the paired 2-tail t test. A difference was considered significant using 95% confidence intervals (p<0.05). Correlation between serum cytokine and serum C-reactive protein titres was performed using simple linear regression analysis incorporating the F test.

RESULTS

Endogenous cytokine production by pancreatic cancer cell lines

Measurement of spontaneous, unstimulated IL-8 production by the 3 human pancreatic cancer cell lines, demonstrated that MIAPaCa2 was a high producer (>1 ng/ml culture medium per 24 hours) of IL-8 (Figure 6.1). Titres of IL-8 increased sequentially with time to a maximum of 50 ng/ml by day 7. The CFPAC cell line demonstrated intermediate spontaneous IL-8 secretion (0.5 - 0.99 ng/ml culture medium per 24 hours)
Production of IL-8 by human pancreatic cancer cell lines at: 0 ( ), 24 ( ), 48 ( ), 72 ( ), 96 ( ), 120 ( ), 144 ( ) and 168 ( ) hours. The adherent human pancreatic cancer cell lines MIAPaCa-2, CFPAC and PANC1 (ECACC) were plated out at a concentration of 5x10^3 cells per well in 96 well tissue culture plates in 200μl of medium per well. Cells were allowed to adhere for 24 hours and then washed using fresh medium prior to incubation. Supernatants were harvested at 24 hour intervals as indicated above. IL-8 and IL-6 measured by ELISA, 2 tailed t-test: *p<0.05, ‡p<0.01, §p<0.005, ¶p<0.001
and the PANC 1 cell line was a low producer of IL-8 (<0.5ng/ml culture medium per 24 hours).

Measurement of basal unstimulated IL-6 production demonstrated that CFPAC cells were high producers of this cytokine (>1 ng/ml of culture medium per 24 hours) (Figure 6.2). The quantity of IL-6 secreted by CFPAC cells increased with time to a peak of 7.5 ng/ml on day 7. The MIAPaCa2 and PANC 1 cell lines secreted insignificant quantities of IL-6.

Although titres of IL-8 in cultures of MIAPaCa2 and CFPAC cell lines increased with time, correction of cytokine production for increasing cell numbers indicated a relatively stable production of IL-8 per cell (Table 6.1). Similarly, IL-6 production by CFPAC cells demonstrated that despite increasing cytokine titre in the culture medium there was a relatively constant rate of cytokine production per cell. (Table 6.1).

Table 6.1

<table>
<thead>
<tr>
<th>Length of culture (hours)</th>
<th>IL-8 per cell MiaPia CaPa2 (pg/cell)</th>
<th>IL-8 per cell CFPAC (pg/cell)</th>
<th>IL-6 per cell CFPAC (pg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0.252</td>
<td>0.110</td>
<td>0.075</td>
</tr>
<tr>
<td>46</td>
<td>0.062</td>
<td>0.016</td>
<td>0.025</td>
</tr>
<tr>
<td>72</td>
<td>0.045</td>
<td>0.023</td>
<td>0.040</td>
</tr>
<tr>
<td>96</td>
<td>0.015</td>
<td>0.053</td>
<td>0.013</td>
</tr>
<tr>
<td>120</td>
<td>0.012</td>
<td>0.003</td>
<td>0.005</td>
</tr>
<tr>
<td>144</td>
<td>0.016</td>
<td>0.003</td>
<td>0.005</td>
</tr>
<tr>
<td>168</td>
<td>0.014</td>
<td>0.002</td>
<td>0.007</td>
</tr>
</tbody>
</table>
Production of IL-6 by human pancreatic cancer cell lines at: 0 (■), 24 (□), 48 (■), 72 (□), 96 (□), 120 (□), 144 (■) and 168 (■) hours. The adherent human pancreatic cancer cell lines MIAPaCa-2, CFPAC and PANC1 (ECACC) were plated out at a concentration of 5x10^3 cells per well in 96 well tissue culture plates in 200μl of medium per well. Cells were allowed to adhere for 24 hours and then washed using fresh medium prior to incubation. Supernatants were harvested at 24 hour intervals as indicated above. IL-8 and IL-6 measured by ELISA. 2 tailed t-test: *p<0.05, ‡p<0.01, §p<0.005, ¶p<0.001
SDS PAGE analysis of supernatants from the Mia PaCa-2 cell line demonstrated a band (Figure 6.3) calculated at molecular weight 8.5 KD which matched exactly that of recombinant IL-8 run in parallel. This band was absent from control runs containing medium only. Further confirmation of IL-8 production was obtained using Western blot analysis which again demonstrated matching bands in the supernatants of MIA PaCa-2 cells and the positive control (medium to which recombinant hIL-8 had been added) (Figure 6.4).
RF versus molecular weight values demonstrating position of putative IL-8 band at 8.5kd. Regression equation $y = -53.159x + 3360.797$, $r^2 = 0.923$, t value 7.741, probability 0.0006.
Figure 6.4
Photograph of Western blot demonstrating band in lane 2 (MiaPaCa-2 supernatant) stained with anti-IL-8 monoclonal antibody. Lane 3 contains medium to which recombinant human IL-8 had been added (positive control), lane 4 contains medium only (negative control), lane 5 contains phosphate buffered saline to which rhIL-8 had been added (positive control). Lane 1 illustrates the positions of the molecular weight markers.
The cDNA amplification product of the IL-8 primers is a single band of the predicted 289 base pair (lane 2). Other lanes contained the predicted 1000 base pair for bcl2 (lane 3) and the 983 base pair for G3PDH (lane 1) which were used as positive controls. IL-6 message was also detected (lane 4). Marker DNA bands are in lane 5.
RT-PCR demonstration of message for IL-8 in the Mia PaCa2 cell line

The cDNA amplification product for the IL-8 primers (Figure 6.5) was a single band of the predicted 289 base pair. Other lanes contained the predicted 1000 base pair for bcl2 and 983 base pair for G3PDH which were used as positive controls. IL-8 message was also detected for CFPAC and PANC1 (data not shown). IL-6 message was detected in the CFPAC and to a lesser extent in PANC1 and Mia PaCa2.

Effect of IL-1, TNF, and IL-4 on IL-8 production by Mia PaCa-2 cells.

Addition of either IL-1 or TNF to 24 hour cultures of Mia PaCa-2 cells resulted in increased concentration of IL-8 in supernatants compared with unstimulated cells (Figure 6.6). The addition of IL-1 and TNF in combination did not result in an increased release of IL-8 by cells compared with that observed with either cytokine added individually. Addition of IL-4 to cells did not alter baseline IL-8 production. Addition of IL-4 and IL-1 in combination resulted in a significant reduction of IL-8 release compared with that observed with IL-1 alone. Similar effects were observed when IL-4 was added in combination with TNF to Mia PaCa-2 cells, however, IL-8 release was still elevated compared with cells to which no cytokine had been added.
Figure 6.6

Effect of IL-1β, TNF-α and IL-4 on IL-8 production by Mia Pia CaPa2 cells in 24 hour cultures. Significance versus 0, * p<0.05, † p<0.01, ** p<0.005, ††, p<0.001

Legend figures denote final concentrations of IL-1β and TNF-α
IL-4 was used in a fixed final concentration of 1 ng/ml.
Effect of IL-1, TNF and IL-4 on IL-8 production by CFPAC cells

Addition of IL-1 to CFPAC cells resulted in increased release of IL-8 only at a concentration of IL-1 of >10ng/ml (Figure 6.7). Addition of TNF also resulted in increased release of IL-8 but again only at concentrations > 10ng/ml of TNF. No dose response curve was evident with either TNF or IL-1 and the addition of both cytokines together demonstrated a similar pattern to addition of either cytokine individually. Addition of IL-4 in combination with either IL-1 or TNF had no suppressive effect on IL-8 release and similarly had no effect on baseline IL-8 production.
Figure 6.7

Effect of IL-1β, TNF-α and IL-4 on IL-8 production by CFPAC cells in 24 hour cultures. Significance versus 0, * p<0.05, † p<0.01, ** p<0.005.

Legend figures denote concentration of IL-1 and TNF in ng/ml final concentration. IL-4 was used in a fixed final concentration of 1 ng/ml.
Effect of addition of supernatants from pancreatic cancer cell cultures on hepatocyte C-reactive protein production

Addition of supernatants from the three pancreatic cancer cell lines, MIAPaCa2, CFPAC and PANC1 to primary cultures of isolated human hepatocytes (Figure 6.8) resulted in significant increases in CRP production. Supernatants from the MIAPaCa2 cell line had the greatest stimulatory effect on C-reactive protein production resulting in 150 ng/ml from a 24 hour hepatocyte culture.

Inhibition of the stimulatory effect of pancreatic cancer cell culture supernatants on hepatocyte CRP production by addition of anti-human IL-8 and anti-human IL-6 antibody.

Figure 6.9 demonstrates that the simultaneous addition of neutralising anti-human IL-8 antibody to hepatocytes in the presence of pancreatic cancer cell supernatants produced partial inhibition of the stimulation of CRP production. This effect was most marked on hepatocytes stimulated with MIAPaCa2 cell culture supernatant. Neutralising anti-human IL-6 antibody had no effect on hepatocyte CRP production in the presence of supernatants from MIAPaCa2 cell cultures. Anti-human IL-6 partially inhibited CFPAC supernatant stimulation of hepatocyte CRP production. Anti-human IL-8 significantly inhibited stimulation of CRP production in response to PANC1 cell supernatants.
Figure 6.8

Effect of pancreatic cancer cell line supernatants on C-reactive protein production by isolated human hepatocytes. Significance versus 0 hrs, * p<0.05, † p<0.01

All supernatants were added in a concentration of 10%

Legend figures denote period of pancreatic cancer cell culture in hours
*p<0.05 vs control
Effect of anti-human IL-8 and anti-human IL-6 neutralising antibody on pancreatic cancer cell supernatant-stimulation of C-reactive protein production by hepatocytes. Significance versus baseline * p<0.05, † p<0.01.

Anti-human IL-8 and anti-human IL-6 neutralising antibodies were used at a concentration of 1 µg/ml.
**Effect of recombinant human IL-8 on hepatocyte CRP production**

The addition of recombinant human IL-8 to hepatocytes resulted in a dose dependent increase in CRP production (Figure 6.10). 1 ng/ml of recombinant human IL-8 increased hepatocyte CRP production by 120% compared with basal production and 10 ng/ml increased CRP production by 150%. Addition of a fixed dose (1ng/ml) of recombinant human IL-6 simultaneously with IL-8 administration demonstrated an additive effect with concentrations of IL-8 greater than 0.1 ng/ml but had no additive effect at lower concentrations. There did not appear to be any synergy between the IL-6 and IL-8 -induction of CRP production. In order to test the purity of recombinant human IL-8 and IL-6 each was tested by ELISA for IL-6 and IL-8 respectively. No cross reactivity was detected between IL-6 and IL-8.

Addition of rhIL-8 also resulted in significant dose dependent increases of α1-antichymotrypsin (Figure 6.11) and α1-acid glycoprotein (Figure 6.12) compared with baseline levels. In this respect IL-8 activity was similar to that observed with rhIL-6. The addition of rhIL-8 and rhIL-6 resulted in significant reductions of production of the negative acute phase proteins prealbumin (Figure 6.13) and transferrin (Figure 6.14). In these experiments no dose dependency was observed in the concentration ranges studied.
Figure 6.10

Effect of recombinant human IL-8 on C-reactive protein production by isolated human hepatocytes. Hepatocytes were plated in wells at a density of $1 \times 10^4$ cells per well. Recombinant human interleukin-8 or interleukin-6 were added (concentrations indicated in the figure) in supplemented William's medium. Supernatants were harvested after 24 hours for analysis of acute phase protein concentration by ELISA. Significance versus 0, * $p<0.05$, † $p<0.01$. 

![Graph showing the effect of recombinant human IL-8 on C-reactive protein production by isolated human hepatocytes.](image-url)
Effect of recombinant human IL-8 on α1-antichymotrypsin production by isolated human hepatocytes. Hepatocytes were plated in wells at a density of $1 \times 10^4$ cells per well. Recombinant human interleukin-8 or interleukin-6 were added (concentrations indicated in the figure) in supplemented William's medium. Supernatants were harvested after 24 hours for analysis of acute phase protein concentration by ELISA. Significance versus 0, * $p<0.05$, † $p<0.01$. 

![Graph showing the effect of recombinant human IL-8 on α1-antichymotrypsin production.](image)
Figure 6.12

Effect of recombinant human IL-8 on $\alpha$1-acid glycoprotein production by isolated human hepatocytes. Hepatocytes were plated in wells at a density of $1 \times 10^4$ cells per well. Recombinant human interleukin-8 or interleukin-6 were added (concentrations indicated in the figure) in supplemented William's medium. Supernatants were harvested after 24 hours for analysis of acute phase protein concentration by ELISA. Significance versus 0, * p<0.05, † p<0.01.
Effect of recombinant human IL-8 on transferrin production by isolated human hepatocytes. Hepatocytes were plated in wells at a density of $1 \times 10^4$ cells per well. Recombinant human interleukin-8 or interleukin-6 were added (concentrations indicated in the figure) in supplemented William's medium. Supernatants were harvested after 24 hours for analysis of acute phase protein concentration by ELISA. Significance versus 0, * $p<0.05$, † $p<0.01$. 

![Graph showing transferrin levels](image-url)
Figure 6.14
Effect of recombinant human IL-8 on prealbumin production by isolated human hepatocytes. Hepatocytes were plated in wells at a density of 1x10^4 cells per well. Recombinant human interleukin-8 or interleukin-6 were added (concentrations indicated in the figure) in supplemented William’s medium. Supernatants were harvested after 24 hours for analysis of acute phase protein concentration by ELISA. Significance versus 0, * p<0.05, † p<0.01.
Effect of recombinant IL-8 on acute phase protein production by HepG2 cells

Addition of rh IL-8 to cultures of Hep G2 hepatoma cells resulted in a significant increase in production of the positive acute phase protein acid 1 glycoprotein (Figures 6.15 a&b). Addition of rhIL-6 resulted in a similar significant increase in AGP production. When IL-8 and IL-6 were added to Hep G2 cells together AGP production was increased to a greater extent than had been observed with either cytokine individually.

The addition of rh IL-8 to HepG2 cells had no effect on haptoglobin production (Figures 6.16 a&b). RhIL-6 produced a dose dependent increase in haptoglobin production. Addition of IL-8 and IL-6 in combination resulted in greater stimulation of haptoglobin production than had been observed with IL-6 alone.

Addition of rhIL-8 to Hep G2 cells stimulate prealbumin production in a dose dependent fashion (Figure 6.17 a&b). This was in contrast to the effect of rhIL-6 which inhibited prealbumin production. Haptoglobin production in response to the addition of IL-8 and IL-6 in combination was dominated by IL-6 induced suppression at low IL-8 concentrations and IL-8 mediated stimulation at concentrations of IL-8 of 1ng/ml and above.
Figure 6.15 a
α1-acid glycoprotein production by HepG2 cells $3 \times 10^4$ cells per well following 48 hour culture.
Cytokines were added at a concentration of 10ng/ml.
Figure 6.15 b
α1-acid glycoprotein production by HepG2 cells 3x10^4 cells per well following 48 hour culture. Cytokines were added at concentrations from 0.1 to 100ng/ml. Significance vs. 0 *=p<0.05, † =p<0.01
Figure 6.16 a
Haptoglobin production by HepG2 cells 3×10⁴ cells per well following 48 hour culture. Cytokines were added at concentrations of 10ng/ml.
Figure 6.16 b
Haptoglobin production by HepG2 cells 3x10^4 cells per well following 48 hour culture. Cytokines were added at concentrations from 0.1 to 100ng/ml. Significance vs. 0 *=p<0.05, †=p<0.01, ††=p<0.01.

Haptoglobin

IL-8  IL-8+IL-6  IL-6
Figure 6.17a

Prealbumin production by HepG2 cells $3 \times 10^4$ cells per well following 48 hour culture. Cytokines were added at concentrations of 10ng/ml.

![Graph showing prealbumin production with bars for 0, IL-8, IL-8+IL-6, and IL-6 conditions. The graph indicates significance levels with p<0.006, ns, and p<0.008 labels.]
Figure 6.17 b
Prealbumin production by HepG2 cells 3x10⁴ cells per well following 48 hour culture.
Cytokines were added at concentrations from 0.1 to 100ng/ml. Significance vs. 0
*= p<0.05, † = p<0.01

![Graph showing prealbumin production by HepG2 cells with cytokine treatments](image)
DISCUSSION

In this study we have demonstrated that human pancreatic cancer cells may constitutively express IL-8, IL-6 or both cytokines. The MIAPaCa2 cell line is a high producer of IL-8 (>1ng/ml) and the CFPAC cell line produces both IL-6 and IL-8. Both of these cell lines produce significant quantities of cytokine but although the titre of IL-8 (Figure 3.1) and IL-6 (Figure 3.2) increased in cell cultures with time the cytokine production per cell remained relatively constant (Table 3.1). This implies the absence of an autocrine inhibitory or stimulatory loop mediated by increasing cytokine concentration, however more detailed analysis of any autoregulatory effects of IL-8 on endogenous cytokine production are required. Recently, it has been demonstrated that IL-8 expression occurs in IL-1 and TNF-stimulated hepatoma cell lines (Sakamoto et al, 1992). There is also evidence that a gastric cancer (Yasumoto et al, 1992), an astrocytoma (Kasahari et al, 1991) and a thyroid carcinoma cell line secrete IL-8 (Yoshida et al, 1992). In these cell lines, however, expression of significant quantities of IL-8 (>1ng/ml) also requires stimulation with either IL-1 or TNF. Stimulation of the MIAPaCa2 cells with either TNF or IL-1 resulted in a further increase in production of IL-8 (Figure ). This demonstrates that although unstimulated production of IL-8 by these cells is high (>1ng/ml), there exists further capacity for the elaboration of this cytokine. The same phenomenon is exhibited by the CFPAC cell line with respect to IL-8 but not IL-6. The failure of MIAPaCa2 cells to produce IL-6 when stimulated with IL-1 or TNF suggests that either the gene, second messenger pathway or the protein synthetic pathway for this cytokine is inactive in this cell line. The failure of either TNF or IL-1 to enhance IL-6 production by CFPAC cells implies that production of this cytokine is regulated by an alternative pathway or that maximal production of IL-6 is already occurring. The PANC 1 cell line was found to be a low producer of IL-8 (<0.5ng/ml/24hours), produced no IL-6 and did not respond to stimulation with either TNF or IL-1.

The addition of IL-4 to cell cultures had no effect on basal cytokine production but reduced both IL-1 and TNF-enhancement of the production of IL-8 by
MIAPaCa2 cells. This finding would imply that IL-4 does not affect cytokine gene expression directly but may down-regulate IL-1 or TNF receptor expression or may interfere with a second messenger pathway involved in IL-1 or TNF signalling. Interferon (IFN)-γ and IL-4 have been shown to down-regulate IL-1-stimulated release of IL-8 by thymic epithelial cells and neutrophils and this emphasises the role of IFN-γ and IL-4 in the regulation of IL-8 expression (Galy & Spitz, 1991; Cassatella et al, 1993; Wertheim et al 1993). IL-4 is normally produced in vivo by lymphocytes. There is evidence in patients with pancreatic and colorectal cancer that T-lymphocyte function is impaired (Falconer et al, 1992, Monson et al, 1986; Balch, 1986). Since it has been demonstrated that IL-4 down regulates IL-6, TNF and IL-1 production by monocytes (Esner et al, 1989; Lee et al, 1990; Fiorentino et al, 1991), improving T cell function might provide a means of enhancing IL-4 production and therefore down-regulating both monocyte and, to some extent, pancreatic cancer cell cytokine production. Neither IL-1 or TNF or the regulatory cytokine IL-4 had a significant effect on IL-8 production by the CFPAC cell line or when they did, only at concentrations which are probably unphysiological.

This study has demonstrated that recombinant human IL-8 stimulates hepatocytes to secrete increased quantities of C-reactive protein (CRP). The validity of this finding is reinforced by the demonstration that supernatants from the MIAPaCa2 cell line, which contain IL-8 but not IL-6, also stimulate hepatocyte CRP production. This did not exclude the possible presence of other cytokines such as Oncostatin M, IL-11 and leukaemia inhibitory factor which are known to regulate production of the acute phase proteins and share common signalling mechanisms with IL-6 (Kishimoto et al, 1993). However, titres of IL-8 in pancreatic cell culture supernatants correlate with doses of recombinant human IL-8 required to produce a given level of CRP production. The inhibitory action of neutralising anti-human IL-8 antibody on the stimulation of hepatocyte CRP production by MIAPaCa2 cell supernatants further supports the observation that IL-8 can induce the production of this acute phase protein. The stimulatory activity of CFPAC supernatants on hepatocyte CRP production was not
inhibited by anti-human IL-8 antibody which was expected since this cell line also produces significant quantities of IL-6.

Recombinant IL-8 also resulted in increased production of the positive acute phase proteins acid 1 glycoprotein and alpha 1 antichymotrypsin in a similar way to the effect of IL-6. IL-8 also suppressed production of transferrin and prealbumin again in a similar way to IL-6. One concern over the use of isolated human hepatocytes to study the effect of potential acute phase protein stimulators is that effects could potentially be mediated through contaminating cells. Although attempts were made to minimise contamination of hepatocyte populations it was decided to study the effects of recombinant IL-8 on a pure hepatoma cell population. Addition of IL-8 to HepG2 cells resulted in increased production of acid 1 glycoprotein in a similar manner to IL-6. IL-8 had no effect on production of haptoglobin whereas IL-6 increased haptoglobin production. Interestingly, IL-8 and IL-6 added in combination produced greater stimulation of haptoglobin than achieved with IL-6 alone. This may suggest that IL-8 may enhance the IL-6 signal by some unknown mechanism. Turning to production of the negative acute phase protein prealbumin by HepG2 cells, IL-8 and IL-6 had quite different effects. IL-6 suppressed prealbumin production and IL-8 stimulated prealbumin production. Addition of IL-8 and IL-6 in combination resulted in prealbumin production rates lying between the levels observed with each cytokine singly. These results suggest that IL-6 and IL-8 probably operate through different signalling mechanisms and raises the possibility that IL-8 may have a role in modifying the IL-6 signal to hepatic acute phase protein production.

The hepatic acute phase protein response (APPR) is a cytokine driven metabolic pathway which has been shown to be elevated in approximately one half of patients with unresectable pancreatic adenocarcinoma (Falconer et al, 1994). Concentrations of hepatocyte-stimulating cytokines might, therefore, be expected to also be elevated in patients with an ongoing APPR. Earlier studies have demonstrated enhanced
production of IL-6 and TNF by peripheral blood mononuclear cells of patients with pancreatic cancer (Falconer et al, 1995). These cytokines are known to stimulate APP production and indeed a correlation was observed between PBMC cytokine production and the presence of a serum APPR as determined by measurement of CRP. In the same study serum TNF was virtually undetected and serum concentrations of IL-6, although elevated in a proportion of patients, did not correlate with the presence of an elevated APPR. IL-8 concentrations have not been measured in the sera of patients with pancreatic cancer. In view of the observations that human pancreatic cancer cell lines produce IL-8 and that this cytokine can stimulate an APPR in isolated human hepatocytes and also in HepG2 cells a study was undertaken to re-evaluate serum concentrations of IL-6 and IL-8 and the acute phase response in patients with pancreatic cancer.
Chapter 7

SERUM INTERLEUKIN-8 AND INTERLEUKIN-6 CONCENTRATIONS IN PATIENTS WITH PANCREATIC CANCER
INTRODUCTION

The hepatic acute phase protein response (APPR) is a cytokine driven metabolic pathway which has been shown to be elevated in approximately one half of patients with unresectable pancreatic adenocarcinoma (Falconer et al, 1994). Concentrations of hepatocyte-stimulating cytokines might, therefore, be expected to also be elevated in patients with an ongoing APPR. Earlier studies have demonstrated enhanced production of IL-6 and TNF by peripheral blood mononuclear cells of patients with pancreatic cancer (Falconer et al, 1995). These cytokines are known to stimulate APP production and indeed a correlation was observed between PBMC cytokine production and the presence of a serum APPR as determined by measurement of CRP. In the same study serum TNF was virtually undetected and serum concentrations of IL-6, although elevated in a proportion of patients, did not correlate with the presence of an elevated APPR.

The previous chapter described the constitutive production of IL-8 and IL-6 by human pancreatic cancer cells and their effect on stimulation of the hepatic APPR in isolated human hepatocytes. Interleukin-8 concentrations have not previously been documented in patients with pancreatic cancer although serum IL-6 concentrations have been measured. The present study investigates serum concentrations of the pro-inflammatory cytokines IL-8 and IL-6 in patients with unresectable pancreatic cancer and their association with the APPR. In addition the relationship between serum cytokine concentrations and resting energy expenditure and parameters marking the progress of cachexia are also investigated.

PATIENTS AND METHODS

Patients

Seventy patients with unresectable pancreatic cancer were studied. The diagnosis was made on the basis of histology or where this was not possible by unequivocal radiological evidence. Tumours were staged according to the UICC
criteria. No patient was jaundiced, had undergone endoscopic stenting or biliary bypass in the 4 weeks prior to study or was receiving cytotoxic chemotherapy or radiotherapy. In addition, at the time of study no patient had clinical evidence of sepsis. Pre-illness stable weight, weight loss, duration of weight loss and body weight were all documented.

**Controls**

Blood samples were also obtained from 18 healthy volunteers for use as controls for measurements of serum IL-6, IL-8 and C-reactive protein.

**Resting energy expenditure**

Resting energy expenditure was measured in fasting rested patients by indirect calorimetry as described in Chapter 2.

**Serum C-reactive protein**

Serum C-reactive protein concentration was measured by ELISA as described previously in Chapter 2.

**Serum IL-6 and IL-8 concentrations**

Serum IL-6 and IL-8 were measured using specific sandwich ELISA's as described in Chapter 2.

**Statistics**

Results are expressed as median and interquartile ranges, associations between groups were analysed by linear regression analysis and analysis of variance.
RESULTS

Patient characteristics, serum CRP concentrations and serum cytokine concentrations are given in Table 7.1. The patients had a median age of 62 years and a distribution of tumour stages similar to other populations studied. Serum CRP was elevated (>10 mg/l) in 40/70 (57%) of patients. The mean serum concentration of CRP was 22.7 mg/l (±2.9 SEM), median 25.1 mg/l.

Table 7.1
Characteristics, nutritional indices and serum IL-6 and IL-8 concentrations and C-reactive protein concentrations of patients with unresectable pancreatic cancer (n=70)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>62 (55-71)</td>
</tr>
<tr>
<td>Tumour stage (per cent)</td>
<td>II 29, III 30, IV 41</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>59 (53-67)</td>
</tr>
<tr>
<td>Pre-illness stable body weight (kg)</td>
<td>70 (59-81)</td>
</tr>
<tr>
<td>Weight loss (kg)</td>
<td>9 (5.8-15.3)</td>
</tr>
<tr>
<td>Resting energy expenditure (KCal/24hrs)</td>
<td>1430 (1210-1658)</td>
</tr>
<tr>
<td>Predicted REE (KCal/24hrs)</td>
<td>1330 (1160-1512)</td>
</tr>
<tr>
<td>Serum C-reactive protein (mg/l)</td>
<td>23.7 (5.4-27.8)</td>
</tr>
<tr>
<td>Serum IL-6 (pg/ml)</td>
<td>103 (0-261)</td>
</tr>
<tr>
<td>Serum IL-8 (pg/ml)</td>
<td>316 (0-1294)</td>
</tr>
</tbody>
</table>

Serum IL-8 was detected in 57% of patients and was present at a concentration of greater than 500 pg/ml in 42.8% (Figure 7.1a). In 32% of patients serum IL-8 concentration exceeded 1000 pg/ml. The mean concentration of IL-8 detected was 846 pg/ml and the highest recorded titre of IL-8 was 7,700 pg/ml. Measurements of IL-8 in sera showed considerable variation and studies were therefore undertaken to assess the reliability of measurements. The results of these investigations are summarised in Table 7.2.
Table 7.2

Variations in the measurement of individual serum samples for IL-8 and IL-6 by ELISA dependent on whether the cytokine concentration was above or below the limit of detection for the assay

<table>
<thead>
<tr>
<th></th>
<th>IL-8</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variation in measurements on individual samples when value was &lt; limit of detection</td>
<td>5%</td>
<td>3%</td>
</tr>
<tr>
<td>Variation in measurements on individual samples when value was &gt; limit of detection</td>
<td>35%</td>
<td>7%</td>
</tr>
<tr>
<td>Correlation between repeated measurements</td>
<td>R=0.76</td>
<td>R=0.93</td>
</tr>
</tbody>
</table>

These results demonstrate that the accuracy of measurements of IL-8 and IL-6 was high for samples at very low or undetectable concentrations of cytokine but that the variability of measurements of serum IL-8 was high at higher concentrations of cytokine. Despite the variability of repeated measurements of IL-8 a consistency was observed (Correlation R=0.76).

Assay of sera for IL-6 demonstrated detectable titres of IL-6 in 65% of patients (Figure 7.1b). Serum IL-6 concentration exceeded 500 pg/ml in only 6% of patients and in 1 patient it was greater than 1000 pg/ml. The mean titre of IL-6 recorded was 177 pg/ml and the highest titre was 1843 pg/ml. 45% of patients had no detectable IL-8 in their sera and 35% had no detectable IL-6.
Figure 7.1

a. Serum concentrations of IL-8 in patients with pancreatic cancer (n=70) and in healthy controls (n=18), p<0.0001 Mann Whitney U test.

b. Serum concentrations of IL-6 in patients with pancreatic cancer (n=70) and in healthy controls (n=18), p<0.0005 Mann Whitney U test.
Figure 7.2

Serum C-reactive protein concentrations in patients with pancreatic cancer (n=70) and in healthy controls (n=18), p<0.0001 Mann Whitney-U test.
None of the 8 healthy controls had detectable serum titres of either IL-8 or IL-6 or CRP.

When the group was studied as a whole a correlation was observed between IL-6 and IL-8 concentrations (R=0.35, p=0.003) (Figure 7.3). Serum IL-8 concentration correlated with serum CRP concentration (R=0.55, p=0.0001) (Figure 7.4) but no such correlation was observed between IL-6 concentration and serum CRP (R=0.03, p=0.83) (Figure 7.5).

Patients were subdivided into those with no APPR (CRP<10 mg/l) n=30, and those who did have an ongoing APPR n=40. Comparison of the two groups demonstrated that they were well matched for age, tumour stage, and body weight. There was no significant differences in total loss of body weight between the two groups, however, patients with an elevated CRP had a greater rate of weight loss than patients with no APPR and this could be explained by their shorter duration of weight loss (APPR 3.5 (3.0-5.0) months vs no APPR 6.0 (3.3-7.5) months, p<0.02). Predicted REE was similar between the two groups but recorded REE was significantly elevated in patients who had an APPR compared with those who did not. There was no significant difference in serum IL-6 concentrations in patients who did and who did not have an APPR. IL-8 concentrations, however, were significantly higher in patients with an elevated APPR compared with those without an APPR (Table 7.3).
Table 7.3

Characteristics, nutritional indices and serum concentrations of IL-8 and IL-6 in patients without an APPR (CRP<10 mg/l n=30) and patients with an APPR (CRP>10 mg/l n=40)

<table>
<thead>
<tr>
<th></th>
<th>CRP&lt;10 mg/l</th>
<th>CRP&gt;10 mg/l</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>62 (55-72)</td>
<td>62 (55-67)</td>
<td>0.9</td>
</tr>
<tr>
<td>SexM:F</td>
<td>20:10</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>Tumour Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>7 (23)</td>
<td>10 (25)</td>
<td>0.47</td>
</tr>
<tr>
<td>III</td>
<td>6 (19)</td>
<td>11 (28)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>18 (54)</td>
<td>19 (48)</td>
<td></td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>63 (54-68)</td>
<td>56 (52-66)</td>
<td>0.16</td>
</tr>
<tr>
<td>Pre-illness stable weight (kg)</td>
<td>75 (65-82)</td>
<td>67 (57-83)</td>
<td>0.14</td>
</tr>
<tr>
<td>Weight loss (kg)</td>
<td>10 (6-19)</td>
<td>9 (6-15)</td>
<td>0.2</td>
</tr>
<tr>
<td>Duration of Weight loss (months)</td>
<td>6.0 (3.3-7.5)</td>
<td>3.5 (3.0-5.0)</td>
<td>0.02</td>
</tr>
<tr>
<td>Rate of weight loss (kg/month)</td>
<td>1.75 (1.0-2.5)</td>
<td>2.5 (2.0-3.3)</td>
<td>0.05</td>
</tr>
<tr>
<td>Predicted REE (KCal/24hrs)</td>
<td>1220 (1100-1450)</td>
<td>1345 (1200-1540)</td>
<td>0.17</td>
</tr>
<tr>
<td>Recorded REE (KCal/24hrs)</td>
<td>1220 (1140-1430)</td>
<td>1605 (1370-1730)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Serum IL-6 (pg/ml)</td>
<td>59.5 (0-221)</td>
<td>140 (22-280)</td>
<td>0.2</td>
</tr>
<tr>
<td>Serum IL-8 (pg/ml)</td>
<td>0 (0-330)</td>
<td>852 (0-1750)</td>
<td>0.0007</td>
</tr>
</tbody>
</table>
Figure 7.3

Correlation between serum IL-6 and serum IL-8 concentrations in patients with pancreatic cancer. $R=0.35$, $p<0.003$ linear regression analysis.
Figure 7.4

Correlation between serum IL-8 and serum CRP in patients with pancreatic cancer. R=0.55, p=0.0001 linear regression analysis.
Correlation between serum IL-6 concentration and serum CRP concentration in patients with pancreatic cancer. R=0.03, p=0.83, linear regression analysis
DISCUSSION

In the present study serum IL-8 was detectable in 57% of patients with pancreatic cancer. It has been suggested that IL-8 could have little activity in vivo because it is almost completely bound to erythrocytes in the circulation. The high affinity for IL-8 by erythrocyte IL-8 binding sites, of which it has been estimated there are $2 \times 10^3$ per cell, is thought to render IL-8 inactive (Darbonne et al 1991). The present investigation has demonstrated significant concentrations of IL-8 in the sera of more than half of the pancreatic cancer patients studied. It is conceivable that in these patients, detection of large quantities of "free" IL-8 represents a saturation of the circulating erythrocyte binding site pool. The implication of this finding is therefore that in these patients excessive production of IL-8 is occurring. Serum IL-6 concentrations were also elevated in two thirds of patients with pancreatic cancer. The correlation between serum IL-6 and serum IL-8 concentrations may indicate some degree of commonality in the mechanisms underlying their release.

The positive correlation between levels of CRP and IL-8 in the sera of pancreatic cancer patients lends some weight to the in vitro findings of an association between this cytokine and induction of the acute phase response. The variability of measurements of IL-8 in sera was high and therefore caution must be exercised in the interpretation of the observed correlation with CRP. Cytokine concentrations measured at a single timepoint represent a view of one instant in time of a process lasting many months. Caution must therefore be exercised in attributing undue clinical significance to serum cytokine concentrations on the basis of simple statistical association. Furthermore, the pattern of induction of cytokines, their tissue of origin and their effect on end-organs is subject to considerable inter-individual variation and is not completely understood. Moreover, it is likely that concentrations of cytokines in the tissue are of far greater significance than circulating levels and it cannot be assumed that serum concentrations bear direct relation to concentrations in the extracellular space.
The aetiology of elevated IL-8 in the sera of patients with pancreatic cancer is obscure. In Chapter 6 the production of IL-8 by human pancreatic cancer cell lines was described. It is possible that the elevated concentrations of IL-8 measured in the present study may reflect tumour IL-8 production however it is equally possible that the IL-8 could have been produced as part of the host-tumour immune response. Further studies using explant technology or RT-PCR of human pancreatic tumour tissue are required to determine whether these tumours produce pro-inflammatory cytokines in vivo.

Weight loss is a common feature of a wide variety of solid epithelial malignancies and contributes significantly to the morbidity and mortality associated with these tumours. Pancreatic adenocarcinomas have a particular propensity to induce cachexia which is not explained solely by reduced nutritional intake. Patients with pancreatic cancer have been shown to have an increased resting energy expenditure and a chronically elevated hepatic acute phase protein response (Falconer et al, 1995). These changes in metabolism account for the reprioritisation of protein synthesis away from muscle and hepatic structural protein toward hepatic acute phase export protein and are thought to be mediated by IL-6, IL-1 and TNF although these factors are frequently not detectable in the sera of patients. It is possible that IL-8 may have a role in the aetiology of the chronic acute phase response in pancreatic cancer patients. This is supported by the observations that recombinant human IL-8 is capable of stimulating acute phase protein production in isolated human hepatocytes (Chapter 6) and that the titres of IL-8 necessary to achieve this phenomenon are equalled or in many cases exceeded by the titres of circulating, "free" IL-8 in patients with pancreatic cancer. Further evidence to support this hypothesis is tendered by the correlation between the presence of elevated titres of serum IL-8 and the presence of an acute phase response in these patients. This hypothesis does not in anyway refute a role for either an inflammatory response to the tumor, sepsis or translocation of endotoxin in the aetiology of the acute phase response in cancer. Many patients with pancreatic cancer have endobiliary stents to relieve their
obstructive jaundice are at increased risk of exposure to endotoxin through cholangitis. Indeed, since bacterial lipopolysaccharide is a potent stimulator of monocyte IL-1 and TNF production in addition to IL-6 and since both IL-1 and TNF enhance the endogenous production of proinflammatory cytokines by human pancreatic cancer cell lines (Chapter 6), it is conceivable that sepsis augments endogenous cytokine production by tumor cells. This may in part explain the fluctuations of acute phase protein levels observed in pancreatic cancer patients within the context of an elevated acute phase response. A hypothesis could be proposed that the chronically elevated acute phase response found in many pancreatic cancer patients may be due in part to endogenous pro-inflammatory cytokine production by tumour cells and that this may be augmented by episodes of sepsis and translocation of endotoxin. These, in turn, result both in monocyte-derived IL-6 stimulation and augmentation of tumour-derived IL-8 and/or IL-6 production with consequent stimulation of hepatocyte acute phase protein production and reprioritisation of the metabolism of the cachectic cancer host.
Chapter 8.

EFFECT OF IBUPROFEN ON ENERGY EXPENDITURE AND ACUTE PHASE PROTEIN PRODUCTION IN PANCREATIC CANCER PATIENTS COMPARED WITH PLACEBO
INTRODUCTION

The cancer cachexia syndrome is complex and involves features such as anorexia, aesthenia, early satiety and hypercatabolism (Fearon, 1992). Although anorexia and malabsorption are important factors contributing to the weight loss observed in patients with pancreatic cancer, the degree of wasting cannot be explained simply by a reduction in nutritional intake. It has been demonstrated (Falconer et al, 1994) that patients with pancreatic cancer have significantly elevated resting energy expenditure and that the most hypermetabolic are those with an ongoing hepatic acute phase protein response (APPR). In Chapter 4 this association between an elevated hepatic APPR and elevated REE was confirmed. Furthermore patients who had an ongoing APPR were also found to have larger deficits in terms of energy intake compared with patients who did not have an APPR. Thus the presence of an elevated APPR is associated with large net energy deficits and this in turn is associated with accelerated weight loss. Therapeutic reduction of the APPR might be associated with reduction in REE and by reducing energy deficit such an approach might be of benefit in the management of cancer cachexia.

The APPR is thought to be mediated by pro-inflammatory cytokines such as TNF and IL-6 (Heinrich, 1990) which in turn have been shown to be capable of mediating a syndrome similar to cancer cachexia in animals (Tracey et al, 1988; Strassmann et al, 1993). Furthermore, infusion of TNF has been shown to increase energy expenditure in man (Starnes et al, 1988). The mediators of the APPR in cancer and their role in producing the variety of metabolic changes associated with cachexia remains unclear. Interleukin-6 , interleukin-1β and tumour necrosis factor-α have all been implicated as potential mediators of the APPR through both direct and prostaglandin-mediated pathways (Heinrich, 1990). Prostaglandins are thought to have an important role in regulating the inflammatory response. The prostaglandin-cytokine axis might be a potential target for therapeutic intervention in patients with cancer cachexia and inhibiting the inflammatory response might result in reductions in both the
hepatic acute phase response and in energy expenditure. Ibuprofen is a non-steroidal anti-inflammatory agent and a potent cyclo-oxygenase enzyme inhibitor which is known to inhibit some of the end organ effects of the pro-inflammatory cytokines. In particular, ibuprofen has been shown to reduce body temperature and the metabolic rate of patients with burn injury (Wallace et al., 1992) and to reduce the level of the acute phase response in some patients with rheumatoid arthritis (Cash et al, 1990). Since there appears to be an association between the acute phase protein response and resting energy expenditure in weight losing pancreatic cancer patients, therapeutic suppression of the APPR might lead to reduction in resting energy expenditure. This study investigates the effect of short term treatment with ibuprofen or placebo on energy expenditure and acute phase protein production in patients with pancreatic cancer.

PATIENTS AND METHODS

Patients

A consecutive series of sixteen patients with histologically proven adenocarcinoma of the pancreas with evidence of weight loss were entered into the study. None of the patients had undergone surgery in the preceding two months. All patients were judged on the basis of clinical evaluation to be free from metabolic or endocrine disorders. None of the patients were jaundiced, pyrexial or had clinical or radiological evidence of infection, or were severely anaemic. In addition, none of the study patients had a history of recent non-steroidal anti-inflammatory drug usage or were taking steroid drugs. All patients had adequate pain control at the time of study. Ten patients were allocated treatment with ibuprofen and values for REE and CRP were compared before and after treatment. To exclude the possibility that either disease progression or familiarity with the method of indirect calorimetry could account for the observed reductions in REE, six patients with pancreatic cancer were given a placebo and measurements of REE and CRP performed before and after treatment. Patients
receiving ibuprofen therapy took 1,200 mg each day in three divided doses for seven days. The six patients received placebo one tablet taken three times each day for seven days. Consent was obtained from each patient and ethical approval was obtained to conduct the trial from the local ethical committee. A group of seventeen healthy subjects, comprising preoperative elective admissions for minor surgery with non-malignant disease, was studied as a control group for comparison with the pancreatic cancer patients.

Measurement of C-reactive protein

Venous blood samples were collected immediately before and after 7 days of treatment with ibuprofen. Serum samples for plasma protein analysis were stored frozen at -70°C until measurement. A sandwich enzyme-linked immunosorbent assay (ELISA) was employed for the measurement of C-reactive protein (CRP) as described in Chapter 2. The limits of sensitivity of the assays, taking into consideration the sample dilutions, were 100 µg/l for CRP.

Nutritional Assessment

Baseline anthropometry, body composition analysis and energy expenditure were assessed as described below in all patients and controls. Nutritional assessment was repeated in the two patient groups following administration for one week of either ibuprofen 1,200mg per day or placebo.

Anthropometry

At the initial assessment, height, pre-illness stable weight and duration of weight loss were recorded. Subjects were weighed on spring balance scales (Seca, Germany) without shoes and wearing light clothing. Ideal body weight was calculated using standardised tables (Metropolitan Life Assurance Tables). Mid upper-arm circumference was measured at the midpoint between the olecranon and acromion processes. Mid-arm muscle circumference (MAMC) was calculated using Jelliffe's
equation. (Jelliffe 1966). Triceps skinfold thickness (TSF) were measured as described previously.

**Body composition analysis**

Multiple frequency bioelectrical impedance analysis (MFBIA) (Xitron 4000 MFBIA Xitron Technologies, San Diego, CA, USA) operated at a current of 200µA root mean square was used to assess body composition. All values were recorded with the subject supine with limbs apart. Repeat measurements were performed using the same pair of limbs. Total body resistance and reactance were taken at 5, 50 and 500 KHz. Values for total and extracellular water spaces were obtained using equations validated in a similar patient group (Hannan et al, 1994). Fat free mass (FFM) was calculated from total body water (TBW) assuming a constant hydration of 73.2% (Pace & Rathburn, 1945).

**Resting Energy Expenditure**

Resting energy expenditure was measured by indirect calorimetry using a ventilated hood system (Deltatrac, S&W Vickers, UK) as described in Chapter 2. Values are expressed per patient and in relation to total body weight and lean body mass. During the course of the study change in weight was incorporated, on an individual basis, in calculations of predicted energy expenditure and body composition analysis.

**Statistics**

Values are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using either a paired or unpaired Student's 2-tailed t test for comparisons between variables and groups as indicated. A p value of less than 0.05 was considered significant.
RESULTS

Patient characteristics

The nutritional status, body composition and resting energy expenditure of the 16 pancreatic cancer patients prior to treatment and of the 17 healthy control subjects are detailed in Table 8.1.

Differences in nutritional indices between cancer and non-cancer patients

In contrast to the weight stable non-cancer controls the pancreatic cancer patients had sustained substantial weight loss (mean: 17% of previous stable weight), with upper arm anthropometry suggesting that both subcutaneous fat and skeletal muscle mass were reduced. Body composition analysis using MFBIA confirmed that the pancreatic cancer patients had a significantly different body composition to that observed in the healthy non-cancer control group. Although total body water was similar between the groups, the cancer patients had significantly lower fat free mass compared with non-cancer patients.

Differences in nutritional indices in patients with pancreatic cancer receiving ibuprofen and placebo

There was no significant difference between the ibuprofen treated and placebo treated cancer patients with respect to age, weight loss, triceps skinfold thickness or body composition. The placebo treated group had a significantly higher mid-arm muscle circumference compared with the ibuprofen treated group (p<0.05).
Table 8.1

Nutritional status and body composition of pancreatic cancer patients (n=16) and healthy non-cancer controls (n=17).

<table>
<thead>
<tr>
<th></th>
<th>PANCREATIC CANCER</th>
<th>HEALTHY CONTROLS</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>60±2.3</td>
<td>56±3.5</td>
<td>NS</td>
</tr>
<tr>
<td>Sex ratio M:F</td>
<td>10:6</td>
<td>12:5</td>
<td>NS</td>
</tr>
<tr>
<td>Weight Kg</td>
<td>58.6±3.8</td>
<td>71.9±3.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Percentage of ideal body weight#</td>
<td>88 ±3.6</td>
<td>108±4.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weight loss as a percentage of previous stable weight</td>
<td>17 ±1.4</td>
<td>nil</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total body water (l)</td>
<td>37.5±1.3</td>
<td>39.5± 1.7</td>
<td>NS</td>
</tr>
<tr>
<td>Fat free mass (kg)</td>
<td>42.8±2.8</td>
<td>52.5±2.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triceps skinfold thickness## (percentage of reference)</td>
<td>64.3 ±4.1</td>
<td>87.5 ±5.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mid arm circumference## (percentage of reference)</td>
<td>91±5.3</td>
<td>102 ±4.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>REE total KCal/24hours</td>
<td>1499±71</td>
<td>1377±58</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>REE KCal/kg body weight</td>
<td>25.58±1.2</td>
<td>19.15±0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>REE KCal/kg fat free mass</td>
<td>35.0±0.9</td>
<td>26.2±0.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NS= not significant (unpaired Student’s t test).

# Metropolitan Life Assurance Tables

## Jelliffe et al .,1966.
Table 8.2
Nutritional status and body composition of the pancreatic cancer patients prior to treatment with ibuprofen (n=10) or placebo (n=6)

<table>
<thead>
<tr>
<th></th>
<th>IBUPROFEN</th>
<th>PLACEBO</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>59 ±2.1ns</td>
<td>62 ±3.5</td>
<td>NS</td>
</tr>
<tr>
<td>Sex ratio M:F</td>
<td>7:3</td>
<td>3:3</td>
<td>NS</td>
</tr>
<tr>
<td>Weight Kg</td>
<td>58.6±3.8ns</td>
<td>58.8±5.6</td>
<td>NS</td>
</tr>
<tr>
<td>Weight loss as a percentage of previous stable weight</td>
<td>16±1.4ns</td>
<td>18±1.9</td>
<td>NS</td>
</tr>
<tr>
<td>Total body water (l)</td>
<td>38.8±2.7ns</td>
<td>35.3±2.5</td>
<td>NS</td>
</tr>
<tr>
<td>Fat free mass (kg)</td>
<td>42.8±2.8ns</td>
<td>43.0±4.0</td>
<td>NS</td>
</tr>
<tr>
<td>Body cell mass (kg)</td>
<td>23.2±2.0ns</td>
<td>22.0±1.5</td>
<td>NS</td>
</tr>
<tr>
<td>Triceps skinfold thickness## (percentage of reference)</td>
<td>62.5±2.7ns</td>
<td>67.0±3.1</td>
<td>NS</td>
</tr>
<tr>
<td>Mid arm circumference## (percentage of reference)</td>
<td>87±6.3*</td>
<td>95±4.2</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

NS= not significant (unpaired Student’s t test).
# Metropolitan Life Assurance Tables
## Jelliffe et al., 1966.

Resting energy expenditure

Measurements of resting energy expenditure demonstrated that the recorded REE for the cancer patients was significantly higher than values for healthy non-cancer subjects (Table 8.1). (mean total REE 1499±71 vs 1377±58 KCal/24hours, p<0.02). Values of total REE (p<0.02), REE per kg body weight (p<0.05) and REE per kg fat free mass (p<0.002) fell significantly from pre-treatment values following 7 days of therapy with ibuprofen 1.2g per day (Table 8.3). In the group treated with ibuprofen, reduction in total REE following treatment resulted in a mean value which was not significantly different from that of healthy controls (REE 1386±88.9 vs 1377±45
KCal/d) (Table 8.3). In the group of patients who received placebo no changes in REE were observed (Table 8.3).

Table 8.3

Recorded resting energy expenditure (REE) values before and after treatment with ibuprofen 1.2g per day or placebo for 1 week

<table>
<thead>
<tr>
<th></th>
<th>IBUPROFEN GROUP (n=10)</th>
<th></th>
<th>PLACEBO GROUP (n=6)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-treatment</td>
<td>Post-treatment</td>
<td>P</td>
<td>Pre-treatment</td>
</tr>
<tr>
<td>REE Total</td>
<td>1468± 98.6</td>
<td>1386±88.9</td>
<td>&lt;0.05</td>
<td>1518 ±61.1</td>
</tr>
<tr>
<td>REE (Kcal/kg BW)</td>
<td>25.62 ±0.9</td>
<td>24.53±0.6</td>
<td>&lt;0.02</td>
<td>25.55 ±0.4</td>
</tr>
<tr>
<td>REE (Kcal/kg FFM)</td>
<td>35.18 ±1.0</td>
<td>33.12±2.4</td>
<td>&lt;0.002</td>
<td>34.83±2.6</td>
</tr>
</tbody>
</table>

Statistical significance versus pre-treatment values NS= not significant (Student’s paired t test).

Serum C-reactive protein concentrations

Serum C-reactive protein concentrations in the patients prior to and following treatment with ibuprofen are shown in Figure 8.1. Measurement of serum C-reactive protein in the ibuprofen group demonstrated concentrations of greater than 100 mg/l in 3 patients prior to commencement of therapy. CRP titres fell in 9 out of 10 patients following treatment with ibuprofen. One patient experienced a marked rise in CRP from 20 to 63 mg/l following treatment and this increase could not easily be explained by sepsis or tissue damage. The mean CRP of the ibuprofen treated group fell from
51±2.4 to 23±1.6 mg/l (p<0.05 2-tailed t test). C-reactive protein was not detected in the sera of any of the healthy control group.
Figure 8.1

Changes in serum C-reactive protein in 10 patients with histologically confirmed carcinoma of the pancreas as determined by ELISA before □ and after □ treatment with 1,200mg of ibuprofen per day for 7 days.
Therapeutic options for pancreatic cancer patients are extremely limited. Approximately 90% of patients have irresectable tumours or metastatic disease at the time of diagnosis (Carter, 1989). Despite recent improvements in diagnosis and staging the prognosis remains very poor with a median survival time of 3-6 months. Patients with adenocarcinoma of the pancreas have among the highest incidence of weight loss of any group of cancer patients which contributes to the morbidity and mortality of this disease (De Wys, 1986). The majority of therapeutic strategies have concentrated on reduction in tumour burden and little attention has been directed toward limiting or reversing cachexia. Clearly the best way to treat cachexia would be to remove the tumour and allow spontaneous recovery of nutritional status (Calman, 1982) but this is rarely feasible (Carter, 1989). Since decline in nutritional status is so closely associated with morbidity and mortality in pancreatic cancer patients, modulation of the inflammatory and catabolic processes which underlie weight loss may offer substantial benefits in terms of duration of survival and quality of life.

This study has confirmed that patients with pancreatic cancer have significantly elevated REE compared with healthy non-cancer subjects (Table 8.1). Treatment with a 7 day course of ibuprofen resulted in a statistically significant reduction of about 6 percent in REE (Table 8.3). In contrast, REE was unchanged during the course of the study in a matched group of pancreatic cancer patients treated with placebo (Table 8.3). The reduction in REE that occurred following treatment with ibuprofen amounted to approximately 80 KCalories per patient per day. Over a six month period, this would reduce a patient's energy deficit by about 14600 Kcal. or the equivalent of about 2Kg of adipose tissue. The long term effects of ibuprofen on REE are not known.

In addition to an elevated energy expenditure, the pancreatic cancer patients had an elevated serum C-reactive protein concentration (mean 51mg/l) when compared
with healthy controls (undetectable). This reinforces the previously described observation of an association between elevation of REE and persistent activation of the hepatic acute phase protein response in weight-losing patients with cancer (Falconer et al, 1994). In the present study, the reduction in REE observed in the ibuprofen treated patients was paralleled by a significant reduction in serum C-reactive protein concentration (Fig 8.1). During semi-starvation the amino acids to support acute phase protein synthesis come from the breakdown of skeletal muscle and it has been pointed out that the amino acid composition of skeletal muscle differs considerably from that of the common acute phase proteins (Reeds et al, 1994). This means that a proportion of amino acids mobilised from skeletal muscle will be oxidised and the transfer of amino acids from one tissue to another will lead to a net loss of nitrogen from the body. Whether the observed attenuation of the acute phase response induced by ibuprofen (Fig 8.1.) might improve significantly the overall nitrogen economy of the wasted cancer patient will require further long term studies.

Ibuprofen is a non-steroidal anti-inflammatory drug with potent inhibitory action on the enzyme cyclo-oxygenase. It is known to inhibit some of the end organ effects of the proinflammatory cytokines IL-6, IL-1 and TNF (Dinarello & Wolff, 1982, Durum et al, 1985). It has been suggested that the pro-inflammatory cytokines IL-6, IL-1 and TNF α, released by cells of the macrophage/monocyte series (Auger & Ross, 1991) may be responsible for the increased energy expenditure and altered nitrogen metabolism that is thought to contribute to weight loss and shortened survival in cancer patients (Fearon & Carter, 1988, Fearon et al, 1991). Recently this hypothesis has been supported by the demonstration that weight losing patients with pancreatic cancer have a chronically elevated hepatic acute phase response and that this is associated with an increased resting energy expenditure compared with healthy controls (Falconer et al, 1994). A further indication of the importance of the presence of an acute phase response in patients with pancreatic cancer is given by the observation that duration of survival is closely associated with the presence or absence of an elevated
serum CRP titre at the time of diagnosis and that this is independent of stage of disease (Falconer et al, 1995).

The mechanism of reduction of acute phase protein production by ibuprofen is uncertain. It might be that ibuprofen reduces production of the pro-inflammatory cytokines such as IL-6, IL-1 and TNF which are known to stimulate acute phase protein production by hepatocytes. It is uncertain whether administration of ibuprofen results in a reduction in circulating cytokines in cancer patients. However there is evidence to suggest that this may be the case in sepsis with pre-treated patients with ibuprofen showing an attenuated TNF response to endotoxin challenge (Spinas et al, 1991; Matrich et al, 1991). Previous studies have demonstrated however, paradoxical elevation of pro-inflammatory cytokine production by isolated peripheral blood mononuclear cells following treatment with ibuprofen (Kunkel et al, 1986; West et al, 1993). Ibuprofen may down regulate acute phase protein production via a prostaglandin mediated pathway resulting in either reduced responsiveness to pro-inflammatory cytokines or via a direct effect on hepatocyte protein production.

This study provides evidence that even relatively short periods of treatment with ibuprofen can significantly reduce both resting energy expenditure and hepatocyte acute phase protein expression. Further studies are required to elucidate whether the use of cyclo-oxygenase inhibitors can alter weight loss over a more protracted period or influence survival. A further study was therefore undertaken to establish the effect of administration of ibuprofen on nutritional and clinical indices.
Chapter 9.

RANDOMISED DOUBLE BLIND, PLACEBO-CONTROLLED TRIAL OF THE EFFECT OF MEGESTROL ACETATE AND IBUPROFEN ON THE PROGRESS OF CACHEXIA IN PATIENTS WITH PANCREATIC CANCER
INTRODUCTION

Cancer cachexia is associated with both reduction of nutritional intake and increased resting energy expenditure. One approach to treating cancer cachexia would be to target both arms of the energy balance equation. Previous work has suggested that megestrol acetate may improve appetite and therefore nutritional intake in weight losing cancer patients. Randomised, placebo controlled trials of heterogeneous groups of cancer patients (mainly hormone-insensitive tumours) have demonstrated that administration of megestrol acetate can produce improvements in weight, appetite and quality of life (Loprinzi et al, 1990, Tchemedyian et al, 1992, Feliu et al, 1991). However, similar studies undertaken in patients with advanced gastrointestinal cancer patients no significant weight gain has been documented (Schmoll et al, 1991, McMillan et al, 1994). In the latter studies a small number of patients did gain weight however response to therapy was unpredictable and that some patients continued to lose weight despite subjective improvement of appetite and objective measurement of increased dietary intake (McMillan et al, 1994). These observations are similar to those described in cachectic patients receiving parenteral nutritional where despite provision of calories excess to dietary requirements only a minority of patients gain lean tissue (von Meyenfeldt et al, 1992). This has lead to the concept that in patients with cancer cachexia; a metabolic block exists to the accretion of lean tissue (Ng & Lowry, 1991).

In Chapter 8 it was demonstrated that oral administration of the non-steroidal anti-inflammatory drug ibuprofen was associated with attenuation of the acute phase protein response and reduction in resting energy expenditure in weight losing patients with pancreatic cancer. In addition previous work has demonstrated that treatment of patients with gastrointestinal cancer with ibuprofen is associated with a reduction in whole body protein kinetics (Preston et al, 1995) and attenuation of serum acute phase protein concentrations (McMillan et al, 1996). These effects might be considered as evidence for correction of the metabolic abnormalities associated with cachexia. The study described in the preceding chapter was a short term investigation and could not
therefore evaluate whether down-regulation of the acute phase response and resting energy results in long term nutritional benefit. The purpose of the present study was to investigate whether such metabolic down regulation which might be achieved though the administration of ibuprofen might allow accretion of lean tissue when combined with the appetite stimulant megestrol acetate.

METHODS

Study design

46 patients with adenocarcinoma of the pancreas were randomised by an independent observer to receive megestrol acetate (Bristol-Myers, Ltd) 480 mg per day in 3 divided doses and either ibuprofen 1,200 mg per day in 2 divided doses or an identical placebo. All of the patients had >5% weight loss, were receiving supportive care and had a life expectancy of at least 2 months. None of the patients had undergone surgery in the preceding two months. All patients were judged on the basis of clinical evaluation to be free from metabolic or endocrine disorders. None of the patients were jaundiced, pyrexial or had clinical or radiological evidence of infection, or were severely anaemic. In addition, none of the study patients had a history of recent non-steroidal anti-inflammatory drug usage or were taking steroid drugs. All patients had adequate pain control at the time of study. Randomisation was performed using study numbers and both patient and observer were blinded to the nature of treatment. The study received ethical approval from the Lothian Ethical committee and written informed consent was obtained from all patients. Patients were assessed before treatment and at 4, 8 and 12 weeks after commencement of therapy.

Measurement of C-reactive protein

Venous blood samples were collected immediately before and at monthly intervals after commencing either megestrol acetate and ibuprofen or megestrol acetate
and placebo. Serum samples for plasma protein analysis were stored frozen at -70°C until measurement. A sandwich enzyme-linked immunosorbent assay (ELISA) was employed for the measurement of C-reactive protein (CRP) as described in Chapter 2.

**Nutritional Assessment**

Baseline anthropometry and body composition analysis were assessed as described below in all patients. Nutritional assessment was repeated in the two patient groups at monthly intervals after commencing either megestrol acetate and ibuprofen or megestrol acetate and placebo.

**Anthropometry**

At the initial assessment, height, pre-illness stable weight and duration of weight loss were recorded. Subjects were weighed on spring balance scales (Seca, Germany) without shoes and wearing light clothing. Ideal body weight was calculated using standardised tables (Metropolitan Life Assurance Tables). Mid upper-arm circumference was measured at the midpoint between the olecranon and acromion processes. Mid-arm muscle circumference (MAMC) was calculated using Jelliffe's equation. (Jelliffe 1966). Triceps skinfold thickness (TSF) was measured as described in Chapter 2.

**Body composition analysis**

Multiple frequency bioelectrical impedance analysis (MFBIA) (Xitron 4000 MFBIA Xitron Technologies, San Diego, CA, USA) operated at a current of 200μA root mean square was used to assess body composition as described in Chapter 2.

**Toxicity assessment**

All patients had blood drawn for full blood count, liver function testing, urea, electrolytes and glucose and serum C-reactive protein at each visit. Toxicity described
by patients or their general practitioners, or observed on clinical examination was also documented.

**Appetite and nutritional intake estimation**

Appetite was assessed using a 10 cm visual analogue scale extending from 0 representing no appetite to 10 representing normal healthy appetite (Raben et al, 1995). Assessment of nutritional intake by patients was assessed using a similar visual analogue scale with 0 representing no intake to 10 representing normal intake i.e. 3 meals per day plus snacks. Objective nutritional intake data was also obtained using 4 day food diaries. Patients were asked to report precise details of food including specific brands and full details of food preparation and method of cooking. Data was analysed using Compeat 4 (Nutrition Systems Ltd, London) software by an independent observer.

**Quality of Life Assessment**

Validated assessments of quality of life and functional ability were completed by patients at each attendance these included the EORTC-QLQ C30 symptom score, EuroQol scale and the Activities of daily living index. In addition Karnofsky performance status was recorded on all subjects.

**Statistics**

Values are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using either a paired or unpaired Student's 2-tailed t test for comparisons between variables and groups as indicated. A p value of less than 0.05 was considered significant.
RESULTS

Patient characteristics

The active group (megestrol acetate and ibuprofen) contained 23 patients of which 13 were males. The placebo group (megestrol acetate and placebo) also contained 23 patients with an identical gender distribution to the active treatment group. Characteristics of patients allocated treatment with megestrol acetate and ibuprofen and megestrol acetate and placebo are given in Tables 9.1 & 9.2. There were no significant differences between the 2 groups with respect to age, tumour stage, weight loss or body weight (Table 9.1). Similarly Table 9.2 demonstrates that there were no significant differences in the baseline anthropometric and body composition values for the two groups.

Table 9.1

Characteristics and pre-study weight loss in patients who received megestrol acetate and ibuprofen and megestrol acetate and placebo.

<table>
<thead>
<tr>
<th></th>
<th>Ibuprofen</th>
<th>Placebo</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex M:F</td>
<td>13:10</td>
<td>13:10</td>
<td>ns</td>
</tr>
<tr>
<td>Age</td>
<td>67 (61-71)</td>
<td>67 (61-75)</td>
<td>ns</td>
</tr>
<tr>
<td>Body weight kg</td>
<td>55.5 (50-62)</td>
<td>57.5 (47.5-64.5)</td>
<td>ns</td>
</tr>
<tr>
<td>Preillness stable weight kg</td>
<td>65.5 (60-79)</td>
<td>66.5 (56.5-79)</td>
<td>ns</td>
</tr>
<tr>
<td>Weight loss kg</td>
<td>11 (8-19)</td>
<td>9 (6.5-20)</td>
<td>ns</td>
</tr>
<tr>
<td>% weight loss</td>
<td>17.5 (13-22)</td>
<td>17 (11-23)</td>
<td>ns</td>
</tr>
<tr>
<td>Duration of weight loss</td>
<td>24 (16-35)</td>
<td>16 (12-24)</td>
<td>ns</td>
</tr>
<tr>
<td>Rate of weight loss kg/month</td>
<td>2 (1-3)</td>
<td>2 (1-3.8)</td>
<td>ns</td>
</tr>
</tbody>
</table>
Baseline anthropometry and body composition in patients who received megestrol acetate and ibuprofen and megestrol acetate and placebo.

<table>
<thead>
<tr>
<th></th>
<th>Ibuprofen</th>
<th>Placebo</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>21 (20-23)</td>
<td>20 (18.7-22.5)</td>
<td>ns</td>
</tr>
<tr>
<td>Pre-illness BMI</td>
<td>25 (23-38)</td>
<td>25 (23-28)</td>
<td>ns</td>
</tr>
<tr>
<td>Arm muscle circumference cm</td>
<td>21.5 (21-24)</td>
<td>21 (18-23)</td>
<td>ns</td>
</tr>
<tr>
<td>Triceps skinfold thickness mm</td>
<td>9 (7.3-10)</td>
<td>8 (5.8-11)</td>
<td>ns</td>
</tr>
<tr>
<td>Total body water l</td>
<td>31.5 (25-36)</td>
<td>29 (23.5-34.8)</td>
<td>ns</td>
</tr>
<tr>
<td>Total body water % of body weight</td>
<td>53 (48-55)</td>
<td>53 (49.3-55)</td>
<td>ns</td>
</tr>
<tr>
<td>Extracellular water l</td>
<td>16 (14-18)</td>
<td>15 (13-18)</td>
<td>ns</td>
</tr>
<tr>
<td>Extracellular water % of body weight</td>
<td>28 (26-29)</td>
<td>28 (25-31)</td>
<td>ns</td>
</tr>
<tr>
<td>Intracellular water</td>
<td>15.5 (11-17)</td>
<td>14 (11.3-17)</td>
<td>ns</td>
</tr>
<tr>
<td>Intracellular water as % of body weight</td>
<td>24.3 (21.8-31.6)</td>
<td>24.4 (20.5-28.8)</td>
<td>ns</td>
</tr>
</tbody>
</table>

Toxicity

Toxicity was similar between the two treatment groups (Table 9.3). There were three principal symptom patterns. Firstly fluid retention manifest as peripheral oedema, ascites and dyspnoea due to pulmonary oedema. Clinical ascites developed in 5 patients receiving active treatment and in 4 receiving placebo, dyspnoea (1 case) and peripheral oedema (1 case) occurred in patients receiving active treatment. The second complication was gastrointestinal haemorrhage this occurred in 2 patients receiving active treatment although neither case resulted in serious clinical deterioration. 1 patient receiving placebo had a fatal haematemesis. The third category of complication was thrombotic disease classified as either deep vein thrombosis in isolation or in association with pulmonary embolus. DVT in isolation occurred in 1 patient receiving active treatment. 2 patients receiving placebo developed bilateral deep vein thromboses associated with pulmonary embolism 1 of these cases resulted in fatality.
Complications arising in patients who received megestrol acetate and ibuprofen and megestrol acetate and placebo during the 12 week study period.

<table>
<thead>
<tr>
<th></th>
<th>Ibuprofen</th>
<th>placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oedema</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Ascites</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Dyspnoea</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>GI haemorrhage</td>
<td>2*</td>
<td>1†</td>
</tr>
<tr>
<td>DVT</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>DVT+PE</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

* 1 haematemesis, 1 melaena neither requiring transfusion
† fatal haematemesis

Quality of Life

Analysis by patient-completed questionnaires of symptom score demonstrated a non-significant increase in symptom score in patients receiving placebo who completed 12 weeks of study. By contrast a significant reduction in symptom score was observed in patients who received active treatment and who completed the 12 week study period (Table 9.4). A disability index was used to assess the difficulty encountered by patients in completing activities of daily living a high score indicating disability. A non-significant increase in disability index was observed in patients receiving placebo who completed the 12 week study period. Statistically significant reduction in disability index was observed in patients receiving active treatment. To attempt to eliminate observer bias subject-determined and observer determined estimates of health status were obtained. No significant differences were observed in either subject or observer determined health status in either group before or after 12 weeks of study. The EORTC quality of life questionnaire was used to assess patients’ quality of life. No significant change in value was observed in the placebo group before and after 12
weeks of study. A significant improvement in quality of life score was observed in patients receiving active treatment who completed 12 weeks of study.

Table 9.4

Quality of life assessments in patients receiving megestrol acetate and placebo or megestrol acetate and ibuprofen

<table>
<thead>
<tr>
<th></th>
<th>Megestrol acetate/placebo</th>
<th>Megestrol acetate/ibuprofen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Symptom score</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EORTC 30 (max 98)</td>
<td>50 (35-72)</td>
<td>54 (36-64)</td>
</tr>
<tr>
<td><strong>Disability index</strong></td>
<td>5 (2-10)</td>
<td>7 (3-9)</td>
</tr>
<tr>
<td>Euroqol (max 14)</td>
<td>6 (3-10)</td>
<td>3.5 (1-8)*</td>
</tr>
<tr>
<td><strong>Subject determined health status VAS</strong></td>
<td>40 (20-70)</td>
<td>50 (30-80)</td>
</tr>
<tr>
<td>(max 100)</td>
<td>45 (15-50)</td>
<td>60 (30-80)</td>
</tr>
<tr>
<td><strong>Observer determined health status</strong></td>
<td>60 (50-80)</td>
<td>65 (50-70)</td>
</tr>
<tr>
<td>Karnofsky performance status (max 100)</td>
<td>60 (50-90)</td>
<td>70 (60-90)</td>
</tr>
<tr>
<td><strong>Quality of life</strong></td>
<td>7 (3-9)</td>
<td>6.5 (3-11)</td>
</tr>
<tr>
<td>EORTC QOL (max 14)</td>
<td>6 (2-12)</td>
<td>10 (6-12)*</td>
</tr>
</tbody>
</table>

Appetite and nutritional intake scores

Measurement of appetite scores demonstrated improvement of appetite in both placebo and active treatment groups from baseline values at 4 weeks (Figure 9.1). Between 4 and 8 weeks a further small improvement in appetite was observed in both groups. Between 8 and 12 weeks appetite scores were maintained in the active treatment group but declined in the placebo group.

Improvements in appetite scores were matched by increase in subjective assessment of patients nutritional intakes from baseline values (Figure 9.1). There were no significant differences in the profile of nutritional intake scores between patients receiving placebo and active treatment. Limited data was available from 4 day food diaries which demonstrated that subjective increase in appetite score for both groups were confirmed by objective measurement of nutritional intake with a 0.6 (-0.2 to +1.0) MJ/day increase in energy intake in the active treatment group and a similar 0.5 (-0.5 to +1.3) MJ/day increase in energy intake in the placebo group.
Changes in appetite and intake scores as assessed by visual analogue scales (VAS) in patients receiving megestrol acetate and placebo or megestrol acetate and ibuprofen. A score of 0 indicates the lowest score of appetite and intake and 9 represents normal healthy appetite and intake.
Weight change

Patients receiving placebo lost a median of 1.5 kg (range -4-4) after 4 weeks of study (Figure 9.2). By 8 weeks the placebo group had lost a median of 2 kg (-5-3) and by 12 weeks they had lost 4.5 kg (-7-0) with only 1 patient remaining weight stable (Figure 9.2). Patients receiving active treatment gained a median of 0.5 kg (range -6-4) after 4 weeks and this weight gain continued with patients gaining a median of 1 kg (range -2-6) after 8 weeks and gaining a median of 2 kg (0-7) by the end of the 12 week study period (Figure 9.2). The difference in weight change was not significant at 4 weeks (p=0.79) but was significant at 8 weeks (p<0.02) and at 12 weeks (p<0.002) (Oldham transformation and linear regression analysis).

Anthropometry

No significant changes in triceps skinfold thickness were observed in patients receiving either placebo or active treatment who returned for assessment at 4, 8 and 12 weeks. Assessment was not possible of non-attenders of which there was a greater prevalence among the placebo group. Similarly arm muscle circumference was not significantly altered from baseline in either group compared with values in attenders at 4, 8 and 12 weeks. However, once again the values for non-attenders could not be obtained.
Changes in weight (kg) in patients receiving megestrol acetate and placebo \( -\) or megestrol acetate and ibuprofen \( -\). Weight at baseline was taken as 0 kg and weight changes plotted subsequently at 4, 8 and 12 week assessments. Difference in weight change was not significant at 4 weeks (\( p=0.79 \)) but was significant at 8 weeks (\( p<0.02 \)) and at 12 weeks (\( p<0.002 \)) (Oldham transformation and linear regression analysis).
Changes in serum C-reactive protein and albumin

The median serum CRP of patients receiving megestrol acetate and placebo was 26 mg/l and this was not significantly different from the median value measured in patients who received megestrol acetate and ibuprofen (Table 9.5). Serum CRP concentrations fell in patients who received megestrol acetate and ibuprofen to a median value of <10 mg/l at 12 weeks however this was not significantly different from baseline values 26 mg/l. No change in serum CRP was observed in patients receiving megestrol acetate and placebo. Serum albumin concentrations were comparable between the two groups at baseline and did not alter significantly during the course of the study.

Table 9.5

Changes in serum C-reactive protein and serum albumin concentrations in patients receiving megestrol acetate and placebo or megestrol acetate and ibuprofen.

<table>
<thead>
<tr>
<th></th>
<th>Megestrol acetate / placebo</th>
<th>Megestrol acetate / ibuprofen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>12 weeks</td>
</tr>
<tr>
<td>CRP mg/l</td>
<td>26 (&lt;10-128)</td>
<td>26 (10-124)</td>
</tr>
<tr>
<td>Albumin g/l</td>
<td>33 (30-40)</td>
<td>33 (28-36)</td>
</tr>
</tbody>
</table>

Survival

Cumulative probabilities for survival are plotted in Figure 9.3. There was no significant difference in median survival between the two study populations megestrol acetate and placebo (median survival 6 weeks) and megestrol acetate and ibuprofen (median survival 9 weeks).
Figure 9.3

Cumulative survival probability from the start of treatment in patients receiving megestrol acetate and placebo — or megestrol acetate and ibuprofen ■. Survival not significantly different between the two groups Log rank test.
DISCUSSION

This study has demonstrated that the combination of megestrol acetate 480 mg/day and ibuprofen 1,200 mg/day is tolerated by patients with advanced pancreatic cancer. Toxicity was acceptable and was not different in profile or incidence between the two groups. Two well recognised side effects of megestrol acetate are fluid retention and venous thrombosis. Although there were a total of 11 cases of clinically evident fluid retention, measurements of total body water and extracellular water suggest that fluid retention was not a great problem. These observations reinforce the observation of Chapter 3 that large volume fluid retention is uncommon except in patients with pancreatic cancer except in those with end-stage malignancy. Thrombotic complications occurred in 3 patients with 2 patients developing subsequent pulmonary embolism. Venous thrombosis is a recognised complication of advanced pancreatic malignancy and the incidence of thrombotic complications is uncertain in this disease with figures between 15 and 80% being quoted in the World literature (Kakkar et al, 1995). Experience would suggest that with the exception of moribund patients the incidence of spontaneous venous thrombosis is substantially lower than published figures and it is uncertain whether the cases observed in the present study were related to the administration of megestrol acetate. Ibuprofen, being a non-steroidal anti-inflammatory drug has a propensity to cause peptic ulceration. Three patients in the present study had evidence of gastrointestinal haemorrhage (2 receiving ibuprofen and 1 receiving placebo). One of these cases, in a patient receiving placebo was fatal. In the present study the majority of patients received concurrent H2 antagonist therapy however there was no association between H2 antagonist administration and GI haemorrhage.

Indices assessing quality of life, severity of symptoms and ability to maintain independent existence were substantially improved in patients receiving megestrol acetate and ibuprofen compared with those patients receiving placebo in whom these indices either remained unchanged or deteriorated. Quality of life assessment is of particular importance in palliative care trials which do not have the potential to make a
major impact on patient survival duration. Indeed in the present study survival although slightly better in patients receiving active treatment was not significantly different from placebo. It is interesting to note however that there was a significantly better reattendance rate for patients receiving active treatment compared with those receiving placebo and this may be related to factors identified in the quality of life assessments such as symptom severity and disability index.

Appetite and nutritional intake scores were improved in both patient groups and these subjective measurements were verified by objective improvement in energy intake. This data concurs with that published in trials of megestrol acetate which have previously documented improvement of these indices. In the present study patients receiving placebo consistently lost weight or at best remained weight stable despite increased nutritional intake. This contrasted with patients reciting active treatment in whom improvement of appetite and nutritional indices were associated with sustained weight gain over the course of the study period. In both groups the rate of weight loss prior to entry to the study was 2 kg per month. Assuming a constant rate of weight loss and assuming that therapeutic intervention was ineffective the predicted weight loss for both groups over the 12 week study period would have been 6 kg. The median weight loss of patients receiving placebo of 4.5 kg is not substantially different from this predicted value however the weight gain observed in the patients receiving megestrol acetate and ibuprofen represents a median net saving of 8 kg.

Previous studies of megestrol acetate have demonstrated weight gain in a variable proportion of patients. One possible explanation for this observation is that improving appetite and energy intake may be effective in only those patients whose principal reason for negative energy deficit is anorexia (see Chapter 7.). By the same reasoning it could be argued that in patients with evidence of hypermetabolism, improving appetite and energy intake in isolation may not be an effective therapeutic strategy. By combining a drug with the potential to modify the inflammatory response
(ibuprofen) with an appetite stimulant this study has demonstrated a more consistent reduction of weight loss and weight gain than previous unipolar strategies. This opens the possibility for further interesting studies such as an investigation of whether ibuprofen improves accretion of lean tissue in patients receiving total parenteral nutrition.
EFFECT OF ORAL SUPPLEMENTATION WITH FISH OIL IN WEIGHT LOSING PATIENTS WITH PANCREATIC CANCER
INTRODUCTION

In Chapter 8 short term administration of ibuprofen; a drug which is known to alter the prostaglandin / cytokine axis; was shown to reduce acute phase protein reduction and resting energy expenditure. In Chapter 9 longer term administration of ibuprofen to weight losing patients with pancreatic cancer was shown to result in attenuation of weight loss and improvement in some aspects of quality of life. Another class of drugs which have shown to be effective in reducing pro-inflammatory cytokine production by isolated peripheral blood mononuclear cells are the n-3 polyunsaturated fatty acids.

It has been suggested that certain polyunsaturated fatty acids (PUFAs) may have a therapeutic role as anti-inflammatory and anti-neoplastic agents. Eicosapentaenoic acid (EPA) has a 20 carbon atom chain containing 5 double bonds (C20:5 n3) and occurs naturally as a major component of fish oil. Gamma-linolenic acid (GLA) has an 18 carbon atom chain containing 3 double bonds (C18:3 n6) and is derived predominantly from seed oils. EPA and GLA and their metabolic precursors linoleic acid (C18:2 n6) and alphalinolenic acid (C18:3 n3) are essential fatty acids and are thought to have an important role in regulating the fluidity of cell membranes (Hwang, 1989) and act as substrates for prostaglandin synthesis (Fisher et al, 1985). The administration of such fatty acids has been associated with alterations in various aspects of the immune and inflammatory response (Hwang, 1989). The mechanisms by which fatty acids exert their immunomodulatory effects are unclear but appear to be diverse.

Administration of PUFAs to healthy volunteers has been shown to down-regulate monocyte derived-interleukin-1 (IL-1) and tumour necrosis factor (TNF) production (Endres et al, 1989) and to inhibit neutrophil and monocyte degranulation, phagocytosis and enzyme release (Virella et al, 1989). Further studies have demonstrated that dietary fish oil inhibits tachycardia and attenuates the maximal increases in temperature and metabolic rate following administration of typhoid vaccine.
Furthermore, IL-1 and IL-6 production following typhoid vaccine administration is suppressed in volunteers treated with fish oil (Cooper et al., 1993). The antipyretic effects of fatty acids cannot however be explained simply by a reduction in IL-1 production, since animals fed with a PUFA-rich diet have an attenuated febrile response to the infusion of IL-1 (Pomposelli et al., 1989). This implies that PUFAs may not only act by modulating monocyte pro-inflammatory cytokine release, but also by changing end-organ responsiveness to cytokine stimulation.

In addition to the anti-inflammatory effects of polyunsaturated fatty acids on cytokine-mediated pathways, there is clinical evidence of immunomodulatory activity resulting from the administration of these drugs, the mechanism of which is uncertain. Cerra et al. (1990) have demonstrated that enteral supplementation containing EPA and other PUFA’s is associated with improvement of immune function and reduction in incidence of infective complications in patients in an intensive care unit setting. It has also been demonstrated that administration of fish oil to patients with rheumatoid arthritis is associated with reduction in joint pain scores and improvement of joint mobility. Further more fish-oil-supplemented enteral nutrition was associated with a 50% reduction in gastrointestinal complications and improvement of renal and hepatic function in post surgical cancer patients (Kenler et al.; 1996).

It has been suggested that one of the major effects of PUFAs in vivo is to modify the synthesis of eicosanoids and prostanoids. Both EPA and GLA have been shown to alter prostaglandin synthesis by competing with arachidonic acid for cycloxygenase and lipoxygenase enzymes. Arachidonic acid is normally converted into the 2 series prostaglandins, the competitive effect of EPA and GLA results in diversion of prostaglandin synthesis away from the 2 series toward the 1 series and 3 series respectively (Fischer et al., 1985). These alterations in prostaglandin synthesis have been considered to be associated with an attenuation of the inflammatory response (Hwang, 1989). It is unclear whether series 1 and series 3 prostaglandins have an anti-
inflammatory effect on human hepatocyte acute phase protein production directly. Essential fatty acid rich diets are associated with a diminution of PGE2 production and the potential benefits of this action are unclear (Ferreti & Flanagan, 1990). PGE2 has been demonstrated to down-regulate monocyte TNF (Kunkel et al, 1988; Spengler et al 1989) and IL-1 production (Kunkel et al; 1986). Furthermore it has been demonstrated that PGE2 appears to have a protective action in the human liver by downregulating Kupffer cell pro-inflammatory cytokine production in an autocrine fashion (Roland et al, 1994; West et al, 1993; Goss et al, 1992) and would thus appear to be a naturally occurring regulator of the inflammatory response. Therefore it would seem unlikely that reducing PGE2 production would have a beneficial anti-inflammatory effect.

The polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and gamma-linolenic acid (GLA) inhibit growth of a variety of human carcinoma cells in vitro (Begin et al, 1985; Begin et al, 1986; Begin et al, 1988; Dippenaar et al, 1982; Karmali et al, 1984; Karmali et al, 1987; Wica et al, 1987) including human pancreatic cancer cell lines (Falconer et al, 1994). Diets supplemented with PUFA inhibit the growth of solid tumours in a variety of tumour-bearing mouse models. In addition, EPA is capable of attenuating the weight loss in the MAC16 murine model of cachexia by a mechanism which appears to be distinct from the anti-tumour effect.

N-3 polyunsaturates have a number of biological activities therefore which might make them effective agents for the treatment of cancer cachexia. This study investigates the effect of dietary supplementation with a complex fish oil (MaxEPA) containing eicosapentaenoic acid, on nutritional parameters and the acute phase response in weight-losing pancreatic cancer patients.
PATIENTS AND METHODS

Patients

18 consecutive patients were studied who had unresectable adenocarcinoma of the pancreas confirmed by histology. No patient had undergone a surgical procedure in the 30 days preceding entry to the study and all had a serum albumin concentration of greater than 30g/litre and a Karnofsky performance score of 70% or greater. Appropriate palliative procedures were allowed but cytotoxic chemotherapy and radiotherapy were excluded. Patients gave written informed consent and the study was approved by Lothian Health Board ethics committee.

Protocol

Patients received 1g soft gelatin capsules containing complex fish oil (MaxEPA®, Seven Seas Health Care, Hull, UK) containing EPA 18% w/w and DHA 12% w/w. Patients were commenced on a dose of 2g/day of fish oil and asked to increase their dosage by 2g at weekly intervals to a maximum dose of 16g/day.

Patients were studied before supplementation, one month after starting supplementation and at a median interval of 3 months (IQR 3-5) from the commencement of supplementation. Each patient therefore acted as his or her own control.

In order to confirm that the observed changes in weight in patients receiving fish oil were not due to a placebo effect, weight loss data were compared with that observed during a previous study using a related PUFA, gammalinolenic acid (GLA). These patients had received a 10 day, continuous intravenous infusion of GLA median cumulative dose 7.6g/day (Scotia Pharmaceuticals, Callanish, UK), administered via a central venous cannula. Following the period of intravenous GLA administration patients were continued on gelatin-encapsulated oral GLA (Scotia Pharmaceuticals,
Callanish, UK) initially at a dose of 3g/day but increasing to a maximum of 6g/day if tolerated.

Patients receiving fish oil underwent the following nutritional assessment before supplementation, at one month from the start of supplementation and thereafter at 2 monthly intervals

**Anthropometry**

At the initial assessment, height, pre-illness stable weight and duration of weight loss were recorded. Mid upper-arm circumference was measured at the midpoint between the olecranon and acromion processes. Mid-arm muscle circumference (MAMC) was calculated using Jelliffe's equation. Triceps skinfold thickness (TSF) was measured using Harpenden calipers (Holtain Ltd, UK). Multiple frequency bioelectrical impedance analysis (MFBIA) (Xitron 4000 MFBIA Xitron Technologies, San Diego, CA, USA) operated at a current of 200μA root mean square was used to assess body composition. Resting energy expenditure was measured by indirect calorimetry using a ventilated hood system (Deltatrac, S&W Vickers, UK) as described in Chapter 2. Values are expressed in relation to total body weight

**Fatty acid extraction and analysis**

Fatty acids were extracted from plasma and erythrocyte membrane phospholipids as described in Chapter 2. This analysis was performed by Prof M.J. Tisdale in the Pharmaceutical Sciences Department of Aston University.

**Measurement of serum C-reactive protein**

An enzyme-linked immunosorbent assay (ELISA) was employed for the measurement of C-reactive protein (CRP) as described in Chapter 2.

**Statistical analysis**
Results are expressed as median and interquartile ranges. Analysis of paired data such as CRP and resting energy expenditure was performed using Student's 2-tailed t test. Data relating to weight loss before and after supplementation with fish oil was expressed as weight change in kg/month and transformed using the Oldham equations prior to regression analysis (Oldham, 1968). Comparisons between weight loss before supplementation in the GLA and fish oil groups were made using a Mann Whitney U test.

RESULTS

Patient characteristics

The characteristics of patients receiving fish oil supplementation are shown in Table 10.1. There were 11 males and 7 females of median age 58 years. The majority of patients (16/18) had advanced disease with stage 3 and 4 tumours. The median weight loss of patients was 16% of pre-illness stable weight.

Table 10.1

Characteristics of patients with pancreatic cancer receiving fish oil supplementation.

<table>
<thead>
<tr>
<th>Age(years)</th>
<th>57 (54-62)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M:F</td>
<td>10:8</td>
</tr>
<tr>
<td>Weight(kg)</td>
<td>63 (52-70)</td>
</tr>
<tr>
<td>% weight loss</td>
<td>16 (9-23)</td>
</tr>
<tr>
<td>Stage of Disease</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2</td>
</tr>
<tr>
<td>III</td>
<td>7</td>
</tr>
<tr>
<td>IV</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are median and interquartile range
Tolerance

Patients receiving fish oil supplement tolerated a median maximum dose of 12g of fish oil/day (IQR 8-15) equivalent to a dose of 2.04 g of EPA/day. No serious toxicity was associated with the administration of fish oil although a number of patients described offensive tasting eructations or transient diarrhoea.

Weight changes

All patients were weight-losing before supplementation (2.9 kg/month (IQR 2-4.6)). Following supplementation with fish oil, a significant change in pattern of weight loss was observed (Figure 10.1), with a median weight gain of 0.3kg/month (IQR 0-0.5) (p<0.002 vs rate of weight loss before supplementation). During supplementation 11 patients experienced weight gain and 3 others became weight stable. 4 patients continued to lose weight but at a reduced rate (median 5kg/month before supplementation versus 2kg/month after supplementation).

Comparison between weight changes following fish oil supplementation and supplementation with gammalinolenic acid (GLA)

There were no significant differences between patients receiving fish oil supplementation or GLA therapy with respect to age, sex or type of palliation (Table 10.2). The GLA group contained 5 patients with stage II disease compared with 2 in the fish oil group. Patients in either group had a similar degree of weight loss and actual body weight. There was no difference between the rate of weight loss in fish oil compared with GLA treated patients before supplementation (fish oil; 2.9 (IQR 4.6-2) vs GLA; 2.9 kg/month (IQR 3.6-1.6), p=0.3 Mann Whitney-U). However, unlike the patients receiving fish oil supplementation (Figure 10.1.), patients receiving GLA continued to lose weight (Figure 10.2.) and there was no significant difference in rate of weight loss in these patients before and after GLA administration (p=0.3).
Weight change before and after a median of 3 months supplementation with fish oil in patients with unresectable pancreatic cancer (n=18). Statistical analysis of changes in weight before and after fish oil supplementation were performed using the Oldham transformation and linear regression analysis.

![Graph showing weight change before and after fish oil supplementation](image)

Difference in rate of weight loss before and at a median of 3 months after commencing fish oil supplementation was significantly different \( p<0.006 \)
Table 10.2.

Characteristics of pancreatic cancer patients receiving either fish oil (n=18) or gammalinolenic acid supplementation (n=20).

<table>
<thead>
<tr>
<th></th>
<th>Fish oil</th>
<th>GLA</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>57 (54-62)</td>
<td>58 (54-63)</td>
<td>ns</td>
</tr>
<tr>
<td>M:F</td>
<td>10:8</td>
<td>12:8</td>
<td>ns</td>
</tr>
<tr>
<td>Total body weight kg</td>
<td>63 (52-70)</td>
<td>64 (54-67)</td>
<td>ns</td>
</tr>
<tr>
<td>Weight loss % pre-illness</td>
<td>16 (9-23)</td>
<td>13 (6-19)</td>
<td>ns</td>
</tr>
<tr>
<td>Stage of disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>II 6</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>7</td>
<td>III 7</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>9</td>
<td>IV 7</td>
<td></td>
</tr>
</tbody>
</table>

Values are median and interquartile range. Comparisons performed using Mann Whitney-U test.
Figure 10.2.

Weight change before and after a median of 3 months supplementation with GLA in patients with unresectable pancreatic cancer (n=20)
For the patients receiving fish oil, changes in weight loss were matched by a significant reduction in serum C-reactive protein concentration median 15 mg/l (IQR 5-24) before supplementation versus 10 mg/l (IQR 4-18) at one month from start of supplementation: p<0.002 (Table 10.3). However this reduction in serum CRP concentration was not maintained and serum concentrations rose to pre-supplementation levels at a median interval of 3 months (IQR 3-5) from commencement of therapy (median 16 mg/l (IQR 10-26) p<0.8 vs 15 mg/l (IQR 5-24) before treatment). Indirect calorimetry demonstrated no overall change in resting energy expenditure when expressed in relation to patients total body weight in the fish oil-supplemented patients (25 Kcal/kg/24 hours (IQR 21-27) before supplementation versus 24 (20-27) at 1 month and 26 (IQR 23-28) at 3 months (Table 10.3). There were however different patterns of response within the group. After one month of supplementation with fish oil, 8 patients had increased REE, the median weight loss in these patients was 0.9kg/month and 9 patients had reduced REE the median weight gain in this group was +0.5kg/month. Regression analysis and analysis of variance demonstrated that increase in resting energy expenditure correlated inversely with weight gain (R=0.61 p<0.008) (Figure 10.3). Bioelectrical impedance analysis showed no significant change in total body water before and during supplementation with fish oil either in absolute terms or expressed as a percentage of total body weight (Table 10.3). Measurements of mid-arm muscle circumference and triceps skinfold thickness similarly demonstrated no significant change from pre-study levels during supplementation (Table 10.3).
Table 10.3

Changes in body composition, energy expenditure and acute phase response in patients (n=18) before fish oil supplementation, one month after supplementation, and at a median of 3 months thereafter

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>1 month</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total body weight (kg)</td>
<td>62 (52-70)</td>
<td>63 (53-72)ns</td>
<td>62 (52-73)ns</td>
</tr>
<tr>
<td>Total body water (l)</td>
<td>37 (28-42)</td>
<td>36 (29-42)ns</td>
<td>34 (29-39)ns</td>
</tr>
<tr>
<td>Total body water as percentage of body weight</td>
<td>56 (49-61)</td>
<td>55 (51-58)ns</td>
<td>55 (50-63)ns</td>
</tr>
<tr>
<td>MAMC (cm)</td>
<td>24 (21-26)</td>
<td>24 (21-26)ns</td>
<td>21 (20-24)ns</td>
</tr>
<tr>
<td>TSF (mm)</td>
<td>11 (7-15)</td>
<td>11 (7-14)ns</td>
<td>10 (6-13)ns</td>
</tr>
<tr>
<td>REE (Kcal/kg/24hours)</td>
<td>25 (21-27)</td>
<td>24 (20-27)ns</td>
<td>26 (23-28)ns</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>15 (5-24)</td>
<td>10 (4-18)†</td>
<td>16 (10-26)ns</td>
</tr>
</tbody>
</table>

Values are median and interquartile range. MAMC: Mid arm muscle circumference, TSF: triceps skinfold thickness, REE: resting energy expenditure, CRP: C-reactive protein. Significance values: Student 2-tailed paired t test vs pre-supplementation values, † p<0.005
Figure 10.3.

Correlation between weight change kg/month and change in resting energy expenditure KCal/kg body weight in patients with pancreatic cancer receiving dietary fish oil supplementation.
The concentration of EPA in plasma phospholipids increased from undetectable levels prior to supplementation to a mean of 5.1±0.7% of the total fatty acids during the course of therapy and DHA increased to a mean of 5.6±0.45% (Figure 10.4). The increase in n-3 fatty acids was paralleled by a decrease in arachidonic from 14.6±1.5% of the total fatty acids prior to fish oil supplementation to 9.0±1.26% after supplementation (Figure 10.4). The changes in plasma phospholipid fractions were paralleled by smaller changes in erythrocyte membrane phospholipid composition (Figure 10.5). There was no evidence for non-compliance. However, the average level of EPA in plasma phospholipids for patients taking 16 capsules per day 4.4±2.2% was somewhat lower than those taking 12 capsules per day 6.0±2.5%, although the difference was not significant (Figure 10.6). The average concentration of EPA for patients taking 8 capsules per day 5.1±1.7% was not significantly different although with 6 capsules the level fell to 1.1%. The relationship between response to supplementation in terms of weight change and declared dosage of MaxEPA taken by patients was studied (Figure 10.6). In general terms a dose response was evident at doses of MaxEPA up to 10g/d. After this there did not appear to be any additional benefit from taking higher doses of MaxEPA. The curve of weight change against dose of maxepa taken does not appear to demonstrate a dose response (Figure 10.7).
Figure 10.4.

Changes in plasma fatty acid composition in patients with pancreatic cancer receiving dietary fish oil supplementation (n=14). Statistical test 2-tailed Student t test.

** p<0.005
†† p<0.001
‡‡ p<0.0001
Changes in erythrocyte membrane fatty acid composition in patients with pancreatic cancer receiving dietary fish oil supplementation (n=14). Statistical test 2-tailed Student t test.
Figure 10.6

Percentage plasma phospholipid fraction EPA in patients according to stated dosage of fish oil taken.
Figure 10.7

Weight change in relation to dosage of fish oil taken

Rate of change of weight
kg/month

Fish oil g/day
DISCUSSION

In this phase II study, oral fish oil supplementation altered significantly the development of cachexia in a group of pancreatic cancer patients. Before supplementation, all of the study group experienced progressive weight loss, however, following administration of fish oil three quarters of the group either became weight stable or actually gained a small amount of weight (Figure 10.1). It is unlikely that the changes observed were due to a placebo effect since administration of the related polyunsaturated fatty acid, gammalinolenic acid, to a matched group of weight-losing pancreatic cancer patients had no influence on the pattern of weight loss (Figure 10.2). That weight stabilisation or weight gain was not secondary to changes in body hydration such as might occur with fluid retention or ascites was demonstrated by the absence of any significant change in the proportion or amount of patient's total body water (Table 10.3). Measurements of mid-arm muscle circumference and triceps skin fold thickness similarly demonstrated no significant change from pre-supplementation levels (Table 10.3), again indicative of stable protein and fat reserves.

It has previously been shown that in patients with pancreatic cancer, those with an ongoing acute phase protein response (i.e. elevated serum C-reactive protein concentration) have enhanced release of pro-inflammatory cytokines from isolated peripheral blood mononuclear cells and are significantly hypermetabolic (Falconer et al 1994). Similarly, Roubenoff and co-workers (1995) have shown a strong correlation between increased resting energy expenditure and enhanced release of pro-inflammatory cytokines from peripheral blood mononuclear cells isolated from cachectic patients with rheumatoid arthritis. The role of pro-inflammatory cytokines in cachexia is further supported by studies in humans indicating that infusion of TNF results in an increased energy expenditure (Starnes et al, 1988) and by studies in animals that demonstrate the development of cachexia following repeated administration of such
cytokines (Tracey et al., 1988) and reversal of cachexia with specific antibodies to the latter (Sherry et al., 1989; Smith & Kluger, 1993).

In the present study after one month of oral fish oil, patients showed a fall in serum C-reactive protein concentration (Table 10.3). Acute phase protein synthesis is known to be strongly influenced by pro-inflammatory cytokines (Heinrich, 1990) and it is possible that the observed fall in C-reactive protein concentration was secondary to the established down-regulatory effects of fish oil on cytokine synthesis by peripheral blood mononuclear cells (Cooper et al., 1993; Endres et al., 1989; Kremer et al., 1990). At a median interval of three months from the initiation of the oral fish oil supplement, the patient's serum C-reactive protein had returned to a level similar to that observed at the beginning of supplementation (Table 10.3). It has previously been shown that in patients with pancreatic cancer, disease progression causes a progressive rise in both the incidence and intensity of the acute phase response (Falconer et al., 1995). Thus, although at the three month time point it would appear that the suppressive effects of the oral fish oil supplement on the acute phase response had been reversed, it is also possible that the level of CRP observed would in fact have been higher had the patients not received fish oil. Overall, median resting energy expenditure was not significantly changed during administration of fish oil (Table 10.3). However, when variation in the response of each patient is taken into account with regression analysis, this demonstrated a significant inverse relationship between resting energy expenditure and weight gain.

In the MAC 16 murine model of cancer cachexia weight loss has been attributed to the production by the tumour of direct catabolic factors acting on muscle and adipose tissue which are different from recognized cytokines. Using this model system it was found that substitution of the carbohydrate component of the diet by fish oil significantly reduced host body weight loss an effect not observed with a GLA-enriched diet. Subsequent studies showed that EPA, but not DHA or GLA was capable of
inhibition *in vitro* of a tumor lipid mobilising factor thought to be responsible for the cachexia and this was confirmed by administration of the pure EPA *in vivo* where weight reversal and re-establishment of the original body weight occurred, an effect not obtained with other PUFAs. In addition to the preservation of host body weight, tumour growth was also significantly inhibited by EPA, but not to a significant extent by DHA or GLA. It is not possible from the present study to determine which component of fish oil is responsible for the effects observed.

In this study weight loss data from fish oil-supplemented patients was compared with that observed in patients receiving supplementation with a related fatty acid GLA. The correct control for trials of dietary fatty acid supplementation is controversial. In previous trials of fish oil in pregnancy and in IgA nephropathy olive oil was used as a control (Olsen et al, 1992; Donadio et al, 1994). Oleic acid, the principal component of olive oil, is not however inert and has been shown to antagonise the anti-tumour effects of n-3 fatty acids *in vitro* (Falconer et al 1994). Like oleic acid, GLA has also been shown to have biological activity and it is conceivable that GLA supplementation might have altered the pattern of weight loss in patients who received it. Nevertheless, in the present study the rate of weight loss was then same before and after supplementation with GLA and therefore it would seem unlikely to have altered the progress of cachexia (Figure 10.2).

That all patients took oral supplementation with fish oil was confirmed by analysis of plasma and erythrocyte phospholipid fatty acid composition (Figures 10.4 & 10.5). In the present study oral supplementation with MaxEPA was associated with significant increases in the phospholipid fraction of EPA and docosahexaenoic acid and with significant reduction in arachidonic acid. This suggests that EPA is adequately absorbed from the gastrointestinal tract in this patient population. The poor correlation between daily dosage of MaxEPA and plasma phospholipid EPA fraction may indicate saturation and β-oxidation or may possibly represent non-compliance with higher daily
dosages (Figure 10.6). The similarity between the dose response curve (Figure 10.7) suggests that the change in weight in this patient population was closely associated with plasma EPA concentration since the profile of this dose response curve almost exactly matches that of the curve of plasma fatty acid phospholipid EPA fraction and dose.

Polyunsaturated fatty acids such as eicosapentaenoic acid have been shown to have an inhibitory effect on human carcinoma cell lines (Falconer et al 1994) and animal tumours in vivo (Beck et al, 1991), it is possible that the effects on the development of cachexia in this study were secondary to an inhibition of tumour growth. Documentation of tumour growth in pancreatic cancer is extremely difficult. Although no patient with evaluable disease showed evidence of a partial or complete response, an effect on tumour growth cannot be excluded. However, in the only other reported trial of fish oil supplementation in human malignancy only 1 partial response was observed in 12 patients with advanced breast cancer (Holyroyde et al, 1988).

A variety of strategies have previously been employed to try and reverse cancer cachexia. Although prednisolone has been shown to improve patient's appetite there was no associated improvement in nutritional status (Willcox et al 1984). Attempts to use either enteral or parenteral nutritional support have at best resulted in an attenuation of the cachectic process with only a minority of patients actually gaining in weight (Cohn et al 1982). These studies demonstrate that targeting nutritional intake in isolation is not an effective therapeutic strategy. In this study fish oil supplementation arrested weight loss and this effect may have been secondary to abrogation of the hepatic acute phase response. The precise mechanism of this effect is unclear. The known effects of EPA on pro-inflammatory cytokine synthesis do present a plausible explanation and this potential mechanism is explored further in Chapter 11.
Chapter 11

EFFECT OF HIGH PURITY ORAL EICOSAPENTAENOIC ACID ON THE PROGRESS OF CACHExIA IN WEIGHT-LOSING PATIENTS WITH PANCREATIC CANCER
INTRODUCTION

In Chapter 10 the results of oral supplementation with fish oil are described in patients with pancreatic cancer. Fish oil supplementation was associated with attenuation of cachexia such that the majority of patients became weight stable or gained weight. The fish oil used in the previous study contained 18% (weight by volume) eicosapentaenoic acid. Although EPA was the major fatty acid component of the fish oil used, a number of other fatty acids notably docosahexaenoic acid 12% (weight by volume) were present and it is possible that the anti-cachectic effects of fish oil could have been due to another fatty acid or a combination of fatty acids rather than EPA per se. The present study was designed to test the hypothesis that EPA was the biologically active component of fish oil responsible for anti-cachectic activity, by using a highly purified preparation of EPA. Furthermore in the previous study patients had taken a median dose of 12g/day of fish oil which was equivalent to 2.16g of EPA. In this study the maintenance dose was 6g of EPA per day and this offered the opportunity to make a crude assessment of whether a dose response effect was evident with a higher dose of EPA.

The mechanism of action of fish oil in attenuating cancer cachexia is unclear. In the study described in the previous Chapter a significant but temporary reduction in serum CRP concentration was observed. Administration of EPA to healthy volunteers has been associated with a reduction in production of IL-1 by peripheral blood mononuclear cells. The purpose of the present study was also to investigate whether administration of high purity EPA to weight-losing patients with pancreatic cancer would down-regulate IL-6 release both in vivo and in vitro from patients' isolated PBMC and whether this might be associated with attenuation of the acute phase response. In addition, by examining the ability of supernatants from patients PBMC to generate an APPR in isolated hepatocytes in the presence or absence of neutralising antibody to IL-6, we wished to determine the importance of IL-6 in the generation of this APPR.
PATIENTS AND METHODS

Patients

A total of twenty-seven patients were recruited to the study. All patients had unresectable pancreatic cancer. Confirmation of diagnosis by histology or unequivocal operative or radiological evidence was obtained in all cases. No patient had previously received radiotherapy or chemotherapy or had undergone surgery or endobiliary insertion of a stent in the preceding 4 weeks prior to entry. On entry to the study patients were registered with the Cancer Research Campaign Data centre who monitored the trial. Written informed consent was obtained from all patients and ethical approval was granted by the Lothian Ethical Committee. Indemnity for the study was provided by the Cancer Research Campaign. None of the patients were jaundiced, pyrexial or had clinical or radiological evidence of infection, or were severely anaemic. In addition, none of the study patients had a history of recent non-steroidal anti-inflammatory drug usage or were taking steroid drugs. All patients had adequate pain control at the time of study. Pancreatic enzyme supplements were administered to patients if they had or developed clinical evidence of steatorrhoea.

Drug formulation and dosage

High purity (95%) EPA, as the free acid, was provided by Scotia Pharmaceuticals Ltd, Callanish, UK, in the form of gelatine capsules. EPA capsules were dispensed in batches of 360 and patients were requested to store them in glass bottles in their refrigerator. The first five patients recruited to the study formed the dose escalation tolerance study group. These patients received EPA 1g per day for the first week, followed by 2g per day for the second week, 4g per day for the third week and 6g per day for the final week. On completing the dose escalation phase these patients received maintenance therapy with Maxepa (Seven Seas) capsules 12-16g per day.
The next 22 patients followed an identical dose escalation phase followed by maintenance therapy with high purity EPA capsules at a dose of 6g per day. These patients were followed up at monthly intervals until death or withdrawal from the study.

**Nutritional Assessment**

Baseline anthropometry, body composition analysis and energy expenditure were assessed as described below in all patients prior to entry into the study. Nutritional assessment was repeated in all patients at monthly intervals following the start of EPA supplementation.

**Anthropometry**

At the initial assessment, height, pre-illness stable weight and duration of weight loss were recorded. Subjects were weighed on spring balance scales (Seca, Germany) without shoes and wearing light clothing. Mid upper-arm circumference was measured at the midpoint between the olecranon and acromion processes. Mid-arm muscle circumference (MAMC) was calculated using Jelliffe's equation. (Jelliffe 1966). Triceps skinfold thickness (TSF) was measured using Harpenden calipers (Holtain Ltd, UK). Three measurements were performed and the mean value recorded.

**Body composition analysis**

Multiple frequency bioelectrical impedance analysis (MFBIA) (Xitron 4000 MFBIA Xitron Technologies, San Diego, CA, USA) operated at a current of 200μA root mean square was used to assess body composition. All values were recorded with the subject supine with limbs apart. Repeat measurements were performed using the same pair of limbs. Total body resistance and reactance were taken at 5, 50, 100, 200, 500 and 1000 KHz. Values for total and extracellular water spaces were obtained using equations validated in a similar patient group (Hannan *et al.*, 1994). Fat free mass (FFM) was calculated from total body water (TBW) assuming a constant hydration of 73.2% (Pace & Rathburn., 1945).
Toxicity assessment

All patients had blood drawn for full blood count, liver function testing, urea, electrolytes and glucose and serum C-reactive protein before commencing EPA and at monthly intervals thereafter. Toxicity described by patients or their general practitioners, or observed on clinical examination was also documented. All toxicity was verified by the trial monitors of the Cancer Research Campaign.

Quality of Life

WHO performance status was recorded on all patients. The hospital anxiety and depression score and Rotterdam symptom scores were used to assess psychological and physical symptoms respectively. Analysis of quality of life questionnaires was performed by an independent clinical psychologist.

Nutritional intake

Nutritional intakes were recorded before and at 2 monthly intervals following the start of EPA supplementation using detailed 4 day food diaries. Patients were requested to measure or weigh food intake and to describe in detail methods of preparation of food. Intakes were calculated by an independent observer using Compute-a diet software.

Survival

Survival was recorded from the time of diagnosis to the time of death. Diagnosis was defined as the date of confirmation of adenocarcinoma of the pancreas by the pathology department or of unequivocal clinical diagnosis in patients in whom definitive pathologic confirmation of diagnosis was lacking. Survival was also documented from the start of treatment to the time of death. Survival is plotted as cumulative probability of survival (Kaplan-Meier analysis) and the number of evaluable patients are recorded beneath the plots.
IN VITRO METHODS

Patients

6 consecutive patients from the main study group were studied before and after receiving a total of 92g of high purity (95%) eicosapentaenoic acid orally, in divided doses over 1 month, in the form of gelatin coated capsules (Scotia Pharmaceuticals, Stirling, UK). These patients formed part of an ongoing phase I trial of eicosapentaenoic acid in pancreatic cancer and met the same inclusion and exclusion criteria as the remainder of the study group.

Isolation of peripheral blood mononuclear cells

Blood samples were obtained from the 6 patients with pancreatic cancer before and after 4 weeks of EPA supplement and from 6 healthy volunteers. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation following layering onto Ficoll as described in Chapter 2. Viable cells were estimated by trypan blue exclusion and were suspended at 1x10^6/ml in supplemented RPMI. The cell suspension was plated at 200µl/well in 96 well flat-bottomed tissue culture plates (Falcon, Becton-Dickinson, Plymouth, UK). Lipopolysaccharide (LPS) from Escherichia coli 0127.B8 (Sigma-Aldrich Ltd, Poole, UK) was added to the appropriate test wells at a final concentration of 10µg/ml (Falconer et al, 1994) and plates were incubated at 37°C in 95% humidified air and 5% carbon dioxide. After 24 hours, supernatants were harvested and stored at -70°C for cytokine analysis and for incubation with hepatocytes.

IL-6 assay

Measurement of IL-6 in supernatants was performed by enzyme-linked immunosorbent assay (ELISA) as described in Chapter 2. The limit of detection was 75pg/ml and interassay variation was 4% (Goldie et al, 1995).
Isolation of human hepatocytes

Human hepatocytes were isolated as described in Chapter 2. Cells were plated at 3x10^4 per well in 96 well flat-bottomed tissue culture plates (Falcon, Becton-Dickinson, Plymouth, UK), pre-coated with rat tail collagen (2μg/well) (Sigma) and were incubated in 95% humidified air 5% CO₂ at 37°C. After 30 hours the hepatocytes were washed with medium in preparation for the addition of supernatants from PBMC cultures.

Incubation of hepatocytes with PBMC supernatants

The supernatants were diluted 1:10 in supplemented William’s medium and added to hepatocytes (200μl/well). Controls of medium only, medium containing IL-6 or medium containing LPS were included. After 48 hours incubation, the supernatants were removed and stored at -70°C for measurement of acute phase protein production. In experiments involving neutralising antibody, monoclonal anti-human IL-6 was added to PBMC supernatants or recombinant IL-6 at a final concentration of 10μg/ml. Controls of medium containing neutralising antibody and medium containing isotype -matched antibody directed against an unrelated antigen were also added to PBMC supernatants.

C-reactive protein assay

The production by hepatocytes of C-reactive protein (CRP) was measured by sandwich ELISA as described in Chapter 2. The lower limit of sensitivity taking into account sample dilutions was 120 pg/ml.

Statistics

Data is presented as mean and standard error of the mean. Statistical analysis was performed using Student’s 2-tailed t-test.
RESULTS

Patient characteristics

The characteristics of individual patients forming the dose escalation and maintenance therapy study groups are shown in Table 11.1. The initial study group comprised 3 males and 2 females of median age 50 years. One patient had a stage 3 tumour and 4 patients stage 4 tumours. The main study group comprised 22 patients of median age 59 of whom 10 were male and 12 female. Tumour stages were as follows: stage 2, 5 patients; stage 3, 7 patients and stage 4, 10 patients. The weight, percentage weight-loss and duration of weight-loss for the initial and main study groups and for patients overall are shown in Table 11.1. At the time of study entry all patients had lost weight (median percentage weight loss 14% (IQR 7-17) compared with pre-illness stable weight). Weight loss had occurred over a median of 4 months (3-6). The median rate of weight loss was 2.0 kg/month (1.4-3.2).

Table 11.1

Patient characteristics at the time of entry to study. Patients 1-5 were the initial study group who were included in the dose escalation regimen of high-purity EPA for a period of four weeks. Patients 6-27 received the same dose escalation regimen and then were maintained on high-purity EPA at a dose of 6g/day.

<table>
<thead>
<tr>
<th></th>
<th>Weight (kg)</th>
<th>Body mass index (kg/m²)</th>
<th>Weight loss (%)</th>
<th>Duration of weight loss (months)</th>
<th>Rate of weight loss (kg/month)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial study group</td>
<td>75.1 (57.6-90.0)</td>
<td>24.2 (23.2-30.2)</td>
<td>15.6 (7.0-17.3)</td>
<td>4.0 (3.5-6.4)</td>
<td>2.7 (1.5-3.6)</td>
</tr>
<tr>
<td>Main study group</td>
<td>66.8 (56.0-75.0)</td>
<td>22.6 (20.6-26.0)</td>
<td>13.0 (7.1-17.5)</td>
<td>4.0 (3.0-5.0)</td>
<td>2.0 (1.4-3.3)</td>
</tr>
<tr>
<td>All patients</td>
<td>67.5 (56.6-76.2)</td>
<td>23.4 (21.2-27.0)</td>
<td>13.9 (7.4-17.4)</td>
<td>4.0 (3.0-6.0)</td>
<td>2.0 (1.4-3.2)</td>
</tr>
</tbody>
</table>

Data presented as median (interquartile range)
Tolerance/toxicity

Full blood count, electrolytes, urea, glucose and liver function tests were assessed at each review. Median values did not change significantly over the course of the study.

The principal symptoms possibly associated with administration of the high purity EPA capsules were nausea and steatorrhoea. No adverse symptoms were noted in the initial study group. Nausea was reported by three of the main study group (CTC grade 1,3 and 3) and was successfully treated with anti-emetics. Steatorrhoea was reported by two of the main study group (WHO grade 1 and 2) and was successfully treated by pancreatic enzyme supplementation.

A number of patients found the high-purity EPA capsules unpleasant to take due to problems with occasional leakage of the contents resulting in an unpleasant chemical taste. Patients were instructed to request replacement capsules if this occurred.

No patients developed adverse events which fell outwith the normal pattern of events for patients with advanced pancreatic cancer and all 24 patients who have died did so due to of progression of their malignancy.

Previous studies with fish oil have indicated a prolonged bleeding time or an alteration in immune function as possible sequelae. One patient had an upper gastrointestinal haemorrhage secondary to duodenal invasion by tumour. This is a well recognised complication of advanced pancreatic cancer. Another patient had a confusional episode shortly before death which was attributed to a cerebrovascular accident. The nature of this event was not resolved and the majority of cerebral events are due to thrombosis or embolism. No other patients experienced clinically evident problems possibly related to either phenomenon.

Changes in body weight

The median values for body weight and rate of weight-loss in the initial and main study groups are shown in Table 11.2. There was no significant change in median
Body weight in either group for patients surviving at each time point up to 24 weeks after commencement of EPA. Median rate of weight-loss decreased significantly in the main study group after 4 weeks of EPA compared with prestudy values. This reduction in the rate of weight loss remained stable thereafter. Individual changes in weight are shown graphically in Figure 11.1.

Table 11.2

Changes in body weight and rate of weight-loss following commencement of EPA. The initial study group underwent dose escalation over four weeks to 6g/day and were then withdrawn from the study. The main study group (n=22) underwent the same dose escalation and then were maintained on high-purity EPA at a dose of 6g/day until death or withdrawal from the study.

<table>
<thead>
<tr>
<th>Duration of EPA supplementation (weeks)</th>
<th>Initial group (n=5)</th>
<th>Main group (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body weight (kg)</td>
<td>Rate of weight change (kg/month)</td>
</tr>
<tr>
<td>0</td>
<td>75.1 (57.6-90.0) [5]</td>
<td>-2.65 (-1.4--3.7)</td>
</tr>
<tr>
<td>4</td>
<td>74 (59.8-95.0) [3]</td>
<td>0 (-0.8--4.4)</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are median (interquartile range)
Figure in squared brackets is number of measurements

a Patient's rate of weight loss compared with baseline p<0.005
Figure 11.1
Changes in body weight of patients (n=22) with pancreatic cancer before and after receiving oral supplementation with high purity EPA. Patients' weight at the time of commencing EPA was taken as zero, pre-illness weight was then extrapolated back and weight change following EPA supplementation was plotted prospectively.
**Anthropometry and body composition analysis**

Median values for MAMC and TSF for the main study group recorded before commencement of EPA and at monthly intervals thereafter are shown in Table 11.3. There was no significant change in either MAMC or TSF in patients surviving up to 24 weeks after commencement of EPA supplementation.

Median values for total body water expressed as a percentage of total body weight are also presented in Table 11.3. Overall, there was no significant change in either variable in patients surviving up to 24 weeks after commencement of EPA supplementation.

Table 11.3

Body composition and anthropometry of patients (n=22) with advanced pancreatic cancer (main study group) who were assessed before commencement of EPA and at monthly intervals thereafter until death or withdrawal from the study.

<table>
<thead>
<tr>
<th>Duration of EPA supplementation (weeks)</th>
<th>Number of patients</th>
<th>Percentage total body water (%)</th>
<th>Triceps skinfold thickness (mm)</th>
<th>Arm muscle circumference (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22</td>
<td>50.5 (46.8-53.1)</td>
<td>11.2 (8.7-13.8)</td>
<td>24.0 (22.9-25.3)</td>
</tr>
<tr>
<td>4</td>
<td>19</td>
<td>48.9 (45.4-52.6)</td>
<td>11.8 (9.4-14.8)</td>
<td>23.5 (21.8-26.1)</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>49.3 (45.7-51.0)</td>
<td>12.3 (7.7-13.5)</td>
<td>23.9 (22.4-25.6)</td>
</tr>
<tr>
<td>12</td>
<td>16</td>
<td>49.3 (46.6-51.2)</td>
<td>12.6 (10.3-14.4)</td>
<td>23.9 (21.9-25.8)</td>
</tr>
<tr>
<td>24</td>
<td>7</td>
<td>51.2 (48.5-54.6)</td>
<td>11.1 (9.3-15.2)</td>
<td>24.0 (22.2-26.0)</td>
</tr>
</tbody>
</table>

Values are median (interquartile range)

No significant difference at 4 weeks or after compared with baseline

**Acute phase protein response**

Changes in serum CRP level with time for individual patients are shown in Figure 11.2. The percentage of patients with an APPR as documented by a CRP ≥10mg/l before commencement of EPA and at monthly intervals thereafter are shown in Table 11.4.
Figure 11.2
Changes in C-reactive protein in patients with pancreatic cancer before and after commencing oral supplementation with high purity eicosapentaenoic acid.
Table 11.4
Proportion of patients with an acute phase protein response as documented by an elevated C-reactive protein (≥10mg/l) of the main study group patients (n=22). Patients had advanced pancreatic cancer and were assessed before commencement of EPA and at monthly intervals thereafter until death or withdrawal from the study.

<table>
<thead>
<tr>
<th>Time from commencement of EPA</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (percentage) of patients with CRP (≥10mg/l) ≥28%</td>
<td>6/21 (28%)</td>
<td>4/16 (25%)</td>
<td>5/16 (31%)</td>
<td>6/15 (40%)</td>
<td>2/8 (25%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CRP : C-reactive protein

**Nutritional intake**

Values for daily caloric intake of 11 patients from the main study group before commencement of EPA and after 4 weeks are presented in Table 11.5. Whilst there was a small increase in calorific intake this difference was not statistically significant.

**Fatty acid analysis**

Values of percentage EPA and AA in plasma phospholipid are shown in Table 11.5. Over 4 weeks of EPA supplementation the percentage of EPA in plasma phospholipids increased significantly while the percentage of AA fell significantly.

**Performance status**

Values for WHO performance status of patients in the main study group are presented in Table 11.5. There was no statistically significant change in performance status with time.
Table 11.5
Values of WHO performance status, daily caloric intake (n=11) and plasma phospholipid EPA and AA levels (n=6) of main study group patients with advanced pancreatic cancer. Assessments were made before commencement of EPA and after 4 weeks. Performance status was noted at monthly intervals.

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO performance</td>
<td>1 (0-1)</td>
<td>1 (0-1)</td>
<td>1 (0-1)</td>
<td>0 (0-1)</td>
<td>0 (0-2)</td>
</tr>
<tr>
<td>status</td>
<td>[22]</td>
<td>[21]</td>
<td>[16]</td>
<td>[15]</td>
<td>[8]</td>
</tr>
<tr>
<td>Daily caloric</td>
<td>1777</td>
<td>1828</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intake (kCal)</td>
<td>(1345-2125)</td>
<td>(1562-2203)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPA in plasma</td>
<td>0</td>
<td></td>
<td>10.0 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>phospholipid (%)</td>
<td>(0-0)</td>
<td></td>
<td>(9.0-14.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA in plasma</td>
<td>8.6</td>
<td>6.1 b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>phospholipid (%)</td>
<td>(5.8-9.2)</td>
<td>(4.8-6.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[6]</td>
<td>[6]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are median (interquartile range)
Figures in squared brackets are number of patients

a Percentage of EPA in plasma phospholipids at 4 weeks compared with baseline p=0.03
b Percentage of AA in plasma phospholipids at 4 weeks compared with baseline p=0.05

No significant differences between performance status at any time point
No significant differences between caloric intake at baseline and 4 weeks
Survival

24 of the total 27 patients have died. 3 patients remain alive at 21, 27 and 53 months from the time of diagnosis. All survivors have a proven histological diagnosis of pancreatic adenocarcinoma. The censored median survival from the time of diagnosis for all patients (n=27) was 216 days (156-414). For the initial study group (n=5) median survival was 167 days (110-398), and for the main study group (n=22) it was 216 days (168-425). The censored median survival from the time of commencing EPA for all patients (n=27) was 181 days (86-334), for the initial study group (n=5) was 41 days (20-206), and for the main study group (n=22) was 188 days (139-339). A plot of the cumulative survival duration of the main study group from the time of commencing EPA is shown in Figure 11.3.
Figure 11.3
Cumulative survival probability in patients receiving high purity EPA (n=22).
This graph demonstrates that prior to commencing EPA all patients had been losing weight and because this plot is based on pre-illness stable weight and duration of weight loss it is assumed that this weight loss occurred at a constant rate. Following commencement of EPA supplementation different patterns of weight change were observed. The majority of patients 16/22 experienced stabilisation of weight or weight gain after the first month. Following this weight was generally maintained with a small number of patients experiencing either modest weight gains or losses. Two patients initially lost weight but then gained weight to greater than pre-supplementation levels. Two patients experienced a marked reduction in body weight following the start of EPA supplementation and continued to lose weight until withdrawal from the study.

RESULTS OF IN VITRO EXPERIMENTS

Effect of EPA on cancer patients serum CRP and IL-6 levels on isolated PBMC IL-6 production rates

The pancreatic cancer patients were 5 males and 1 female of mean age 58 years ± 5. Patients had lost a mean of 12.7% ± 3.2 of pre-illness stable weight over a mean period of 7.3 months ± 1.7 prior to diagnosis. The mean serum CRP concentration in patients with pancreatic cancer was 11.0 mg/l ± 4.8 (Figure 11.4). Following supplementation with EPA, CRP concentrations fell significantly to a mean of 0.8 mg/l ± 0.8 (p<0.05). Before EPA supplementation the mean serum IL-6 concentration was 687 pg/ml ± 320, following supplement this had reduced to 292 pg/ml ±99, however, this
Figure 11.4
Serum C-reactive protein (CRP) concentrations and interleukin-6 (IL-6) concentrations in patients with pancreatic cancer (n=6) before and after receiving a total of 92g of oral eicosapentaenoic acid over 1 month.

*p<0.05, ns=not significant Post EPA versus Pre EPA, Student 2-tailed paired t-test
difference was not statistically significant (Figure 11.4). Neither CRP nor IL-6 could be detected in the sera of healthy controls (data not shown). Before commencing EPA supplementation, spontaneous and LPS-stimulated production of IL-6 by peripheral blood mononuclear cells isolated from pancreatic cancer patients was significantly increased compared with healthy controls (Figure 11.5). Following 4 weeks of EPA supplementation, IL-6 production by PBMC’s had fallen in the cancer patients to levels which were not significantly different from those observed in the untreated healthy controls.

Effect of recombinant IL-6 and IL-6 neutralising antibody on CRP production by isolated human hepatocytes

Addition of recombinant human IL-6 to hepatocytes resulted in significant dose dependent increases in CRP production by hepatocytes (Figure 11.6). Addition of neutralising anti-IL-6 antibody abrogated the effect of IL-6 in stimulating CRP production (Figure 11.6). LPS and isotype-matched antibody directed against an unrelated antigen had no effect on hepatocyte CRP production.

Effect of EPA on the ability of PBMC supernatants from cancer patients to stimulate CRP production by isolated human hepatocytes.

Production of CRP by isolated human hepatocytes in response to the addition of supernatants from unstimulated or LPS-stimulated PBMC isolated from cancer patients prior to receiving EPA supplementation, was significantly greater than production of CRP in response to supernatants from PBMC isolated from healthy controls (Figure 11.7). Following one month of oral supplementation with EPA, production of CRP in response to the addition of supernatants from unstimulated or LPS-stimulated PBMC, was reduced compared with pre-supplementation levels.
Effect of neutralising antibody to IL-6 on the ability of PBMC supernatants to stimulate CRP production by isolated hepatocytes.

Addition of neutralising anti-IL-6 antibody to supernatants from PBMC derived from either cancer patients or healthy controls had no effect on the ability of supernatants from PBMC cultured in the absence of LPS to stimulate CRP production (Figure 11.8). The ability of supernatants from LPS-stimulated PBMC to induce CRP production by hepatocytes was significantly inhibited by the addition of anti-IL-6 antibody both in controls and in cancer patients prior to EPA supplement. Addition of anti-IL-6 antibody had no further suppressive effect on the potential of LPS-stimulated PBMC, isolated from patients who had received oral EPA supplementation, to stimulate CRP production by hepatocytes (Figure 11.8).
Figure 11.5
Production of IL-6 by peripheral blood mononuclear cells (PBMC) isolated from healthy controls and patients with pancreatic cancer before and after 4 weeks oral supplement with EPA (total dose 92 g). PBMC were cultured in the presence and absence of 10μg/ml LPS, supernatants were harvested after 24 hours and assayed for the presence of IL-6 by ELISA.

* p<0.05 versus healthy controls † p< 0.05 versus pancreatic cancer before EPA, Student 2-tailed paired t-test.
Figure 11.6

Effect of recombinant IL-6 on C-reactive protein (CRP) production rates by isolated human hepatocytes in the presence and absence of anti-human IL-6 neutralising antibody (10µg/ml).

\[\text{CRP ng/ml}\]

\[\text{IL-6 concentration ng/ml}\]

\[\dagger p<0.01, \dagger\dagger p<0.001\] IL-6 versus 0 control, Student 2-tailed paired t-test.
Figure 11.7
C-reactive protein (CRP) production by isolated human hepatocytes in response to PBMC supernatants from healthy controls and patients with pancreatic cancer before and after 4 weeks of receiving oral EPA (92g total). PBMC supernatants were added to isolated human hepatocyte cultures at a concentration of 1:10. Following 48 hour incubation hepatocyte supernatants were harvested and assayed for acute phase proteins.

![Graph showing CRP production](image)

* * p<0.05 versus healthy controls † p < 0.05 versus pancreatic cancer before EPA, Student 2-tailed paired t-test.
Effect of EPA on the ability of PBMC supernatants from cancer patients to stimulate production of other acute phase proteins by isolated human hepatocytes.

Concentrations of the other positive acute phase proteins studied; alpha 1 antichymotrypsin (Figure 11.9) and acid 1 glycoprotein (Figure 11.10); were also reduced compared with pre-supplementation levels however this reduction was only significant in the case of alpha 1 antichymotrypsin. Production of the negative acute phase proteins prealbumin (Figure 11.11) and transferrin (Figure 11.12) was both increased in response to PBMC supernatants from cancer patients following EPA supplementation compared with pre-supplement levels.

Effect of EPA on incorporation of $^3$H thymidine by lymphocytes in the presence and absence of phytohaemagglutinin.

Incorporation of thymidine by lymphocytes of patients with pancreatic cancer was significantly lower than that observed in controls both in unstimulated cultures and in cultures which had been stimulated with the T-cell mitogen phytohaemagglutinin (Figure 11.13). Following EPA supplementation an increase in incorporation of thymidine was observed both in PHA-stimulated and in unstimulated lymphocyte cultures such that thymidine incorporation was not significantly different from that observed in cultures isolated from healthy controls.
Figure 11.8
Effect of anti-human IL-6 neutralising antibody on C-reactive protein (CRP) production by isolated human hepatocytes in response to PBMC supernatants from healthy controls and patients with pancreatic cancer before and after 4 weeks of receiving oral EPA (92g total). PBMC supernatants were added to isolated human hepatocyte cultures at a concentration of 1:10. Following 48 hour incubation hepatocyte supernatants were harvested and assayed for CRP.

* p<0.05 Versus LPS for each group, Student 2-tailed paired t-test.
α1-antichymotrypsin production by isolated human hepatocytes in response to PBMC supernatants from healthy controls and patients with pancreatic cancer before and after 4 weeks of receiving oral eicosapentaenoic acid (92g total). PBMC supernatants were added to isolated human hepatocyte cultures at a concentration of 1:10. Following 48 hour incubation hepatocyte supernatants were harvested and assayed for α1-antichymotrypsin.

![Graph showing α1-antichymotrypsin production](image)

- Controls
- Before EPA
- EPA

- □ Spontaneous
- ■ LPS

*Statistically significant difference
α1-acid glycoprotein production by isolated human hepatocytes in response to PBMC supernatants from healthy controls and patients with pancreatic cancer before and after 4 weeks of receiving oral eicosapentaenoic acid (92g total). PBMC supernatnats were added to isolated human hepatocyte cultures at a concentration of 1:10. Following 48 hour incubation hepatocyte supernatants were harvested and assayed for α1-acid glycoprotein.
Figure 11.11

Prealbumin production by isolated human hepatocytes in response to PBMC supernatants from healthy controls and patients with pancreatic cancer before and after 4 weeks of receiving oral eicosapentaenoic acid (92g total). PBMC supernatants were added to isolated human hepatocyte cultures at a concentration of 1:10. Following 48 hour incubation hepatocyte supernatants were harvested and assayed for prealbumin.
Transferrin production by isolated human hepatocytes in response to PBMC supernatants from healthy controls and patients with pancreatic cancer before and after 4 weeks of receiving oral eicosapentaenoic acid (92g total). PBMC supernatants were added to isolated human hepatocyte cultures at a concentration of 1:10. Following 48 hour incubation hepatocyte supernatants were harvested and assayed for transferrin.
Figure 11.13

T-cell blastogenesis in healthy controls and pancreatic cancer patients before and after 4 weeks of oral eicosapentaenoic acid supplement (total 92g). PBMC were isolated and cultured in the presence and absence of phytohaemagglutinin (PHA) 100μg/ml for 72 hours. Cells were then pulsed with 1μCi tritiated thymidine and incorporation measured after 2 hours by scintillography.

![Graph showing T-cell blastogenesis before and after EPA supplement](image-url)
Figure 11.14
Changes in the concentrations of eicosapentaenoic acid (EPA) and arachidonate in the plasma of patients measured by gas chromatography before and after 4 weeks of receiving oral EPA supplementation expressed as percentage of total plasma phospholipid fatty acids.
DISCUSSION

Oral supplementation with high purity EPA altered significantly the progress of cachexia in this group of patients with advanced pancreatic cancer. Before supplementation all patients had lost a substantial amount of weight (median 14% of pre-illness weight). After supplementation there was no further significant decline in weight. The rate of weight change was reversed from a median loss of 2kg/month before supplementation to a median gain of 1kg/month in the main study group after 4 weeks. After this time the rate of weight change remained static suggesting a stabilisation of weight. This attenuation of cachexia in advanced pancreatic cancer is similar to that seen in Chapter 10 following oral supplementation with a crude fish oil preparation and contrasts with the weight change expected in patients with advanced pancreatic cancer given full supportive care alone who progressively lose weight until death (Chapter 3). Thus EPA appears to be effective in attenuating cachexia and may be one of the principal components of fish oil responsible for anti-cachectic activity. In the previous study described in Chapter 10, patients taking fish oil received an effective daily dose of EPA of 2.2g. The increment to 6g/day in the current study does not appear to have been associated with any greater anti-cachectic effect since in both studies patients essentially became weight-stable.

It has been suggested that increased body weight following nutritional support in cancer patients is largely due to the accumulation of body water (Bozetti 1992). However, the weight-stabilisation seen in the current study was not due to changes in hydration since there were no significant changes in total body water or total body water as a percentage of body weight throughout. Measurements of MAMC and TSF similarly demonstrated no significant change from baseline levels suggesting stabilisation of protein and fat reserves with EPA. Again, this is in contrast to the continuing decline in MAMC and TSF seen in similar patients who undergo no specific intervention (Chapter 3).
One mechanism whereby EPA might attenuate the progress of cachexia is via a reduction in pro-inflammatory cytokine release. In the subgroup of the present study population (n=6) down-regulation of pro-inflammatory cytokine release followed the initial one month dose-escalation period with EPA. Prior to EPA supplementation PBMC supernatants from cancer patients stimulated CRP production by isolated hepatocytes to a significantly greater level than PBMC supernatants from healthy controls. This effect could be largely inhibited by the addition of anti-IL-6 antibody suggesting that IL-6 is the dominant cytokine stimulating the acute phase protein response in these supernatants. EPA supplementation also reduced the ability of PBMC supernatants to stimulate CRP production by isolated human hepatocytes. Further confirmation of the role of EPA in suppressing IL-6 production was obtained from the observations that the ability of PBMC supernatants from cancer patients to elicit CRP production could not be inhibited further following EPA supplementation. CRP may be used as a marker for pro-inflammatory cytokine activity in vivo. In the present cohort of patients, the proportion with an elevated CRP remained stable at between 25 and 40% throughout the study period. That there was no consistent reduction in CRP levels could be explained by the fact that only 28% of patients had a CRP ≥10mg/l prior to study entry. Defining an APPR as a CRP level ≥10mg/l is somewhat arbitrary and it is possible that there were patients whose CRP started below this concentration and decreased further but this would not be detected by our standard turbidometric assay. Individual patients did, however, demonstrate transient increases in CRP levels. These episodes sometimes corresponded with periods of cholangitis whereas in other instances there was no clinically evident cause. In patients with advanced pancreatic cancer receiving no specific intervention CRP tends to rise with disease progression (Falconer et al 1994). Whether there was a genuine suppression of the incidence or intensity of the APPR associated with long-term EPA administration in the present study is not clear. A prospective randomised trial and a more sensitive CRP assay would be required to address this question.
The mechanism of weight-loss in pancreatic cancer is thought to involve both anorexia and hypermetabolism (Chapter 4). Patients may become anorectic due to clinically obvious causes such as direct tumour invasion of the gastrointestinal tract. However, it is likely that other factors are involved including pro-inflammatory cytokines. In the present study there was a small but non-significant rise in the calorie intake in the subgroup of patients who were assessed during the initial 4 week period of EPA dose escalation. Whether this contributed to weight stabilisation and whether this level of food intake continued throughout the study is not known. However, the median value of around 1800kcal/day is certainly compatible with maintenance of weight stability in a relatively elderly and sedentary group of patients.

Polyunsaturated fatty acids such as EPA have been shown to have an inhibitory effect on human pancreatic carcinoma cell lines in vitro (Falconer et al, 1994b). These effects may occur via cell cycle arrest and the induction of apoptosis (Lai et al, 1996). EPA will also slow the growth of experimental tumours in mice (Beck et al, 1991) and it is possible that the arrest of cachexia in the present study was secondary to inhibition of tumour growth. However, serial tumour imaging in pancreatic cancer is difficult to interpret and expensive and was not performed in the current study. EPA (but not the related n-3 fatty acid DHA) also inhibits the effects of tumour associated proteolytic and lipid-mobilising factors (Tisdale, 1996). Todorov et al (1996) have recently characterised such a proteolytic factor associated with weight-loss in cancer patients but not those losing weight secondary to trauma or sepsis. The latter studies provide an alternative mechanism whereby EPA may attenuate cachexia without affecting tumour growth.

No change in performance status was observed after EPA supplementation for 24 weeks. Quality of life generally deteriorates with time in patients with advanced cancer and performance score has been found to be the strongest factor predicting overall quality of life (Mor, 1987). It is, therefore, possible that EPA modulated the expected progressive deterioration in quality of life of patients over the study period.
Overall survival of patients with pancreatic cancer is very poor with a median of 4.1 months (Ahlgren, 1996). Median survival in this study was 7.2 months but a condition of enrolment was that survival was expected to be over 2 months. Similar conditions apply to most chemotherapy trials where median survival of untreated patients has been noted to be between 63 and 122 days and overall median survival of treated patients to be between 160-170 days (Fearon et al, 1996). Clearly overall survival in this study is at the upper end of that seen in chemotherapy trials but without the side-effects associated with chemotherapy.

In this phase I/II study the initial study group (n=5) underwent escalation of high purity EPA as the free acid to a dose of 6g/day without major intolerance or toxicity. In the main study group (n=22), dose escalation and subsequent maintenance at a dose of 6g/day EPA as the free acid were similarly well tolerated. There was a marked rise in levels of EPA in plasma phospholipids from being undetectable before, to representing around 10% of fatty acids after 4 weeks of EPA supplementation and this provided an index of patient compliance. This rise was accompanied by a fall in AA. Such a pattern of change is similar to that previously reported following fish oil supplementation (Leaf and Weber, 1989, Chapter 10). However, the percentage of EPA incorporation achieved is roughly twice that seen after supplementation with crude fish oil capsules in pancreatic cancer patients given a final dose equivalent to 2g EPA/day after a similar dose escalation period Chapter 10). In the present study the patients who had an interruption of EPA supply (n=13) and who took fish oil capsules to fill in the gap did not appear to follow a different clinical course and maintained their weight in a similar fashion to the group who received EPA alone (rate of weight-change at 3 months : EPA alone - median +0.4kg/month (+0.2-+0.6)(n=9) versus EPA/Maxepa - median +0.1kg/month (-0.2--+0.2)(n=13); p=0.32). This would tend to confirm the concept that, as far as the anti-cachectic activity of EPA is concerned, an increase from 2g/day to 6g/day does not confer additional benefit.

EPA and fish oils have been suggested to have a variety of adverse effects. Patients with pancreatic cancer tend to be insulin resistant (Cersosima et al, 1991) and
fish oil supplementation has been found to adversely affect glucose metabolism in otherwise well patients with non-insulin-dependent diabetes mellitus (Glauber et al, 1988, Borkman et al, 1989). No clinically significant, persistent rise in random blood glucose levels was observed in this study. n-3 fatty acids have been reported as prolonging bleeding time and inhibiting platelet function raising concerns about excessive bleeding after consuming fish oils (van Howelingen et al, 1987). However, only one study has found this to be a clinically significant problem with an increased rate of epistaxis in hyperlipidaemic adolescents given fish oil (Clarke et al, 1990). Two patients in this study suffered adverse events conceivably related to bleeding but while bleeding time was not measured, platelet counts were maintained well within the normal range. There has been concern that the immunomodulatory effects of fish oils (Calder et al, 1990) may result in dangerous immunosuppression (Leaf and Weber, 1989) but to our knowledge this has not been reported and there were no obvious infective complications in this study. In addition the suppressive effect of EPA on lymphocyte proliferation which has been described in animal studies (Calder et al, 1990) was not observed in the present study (Figure 11.13).

This study shows that EPA would appear to be a safe, effective anti-cachectic agent. A randomised, controlled trial would be required to consolidate the observed anti-cachectic effect, evaluate any effect on survival and reveal any as yet undetected side-effects. Further study is required to determine if nutritional supplementation in combination with EPA will result in weight-gain rather than weight-stability and whether large doses of EPA will have direct anti-tumour effects in human malignancy.
GENERAL DISCUSSION

Cachexia is a common sequela of gastrointestinal and other malignancies. The high incidence of malignancies which are associated with weight loss make inhibition of cachexia an important therapeutic goal. Weight loss and consequent lean tissue depletion is considered to give rise to much of the morbidity and mortality associated with advanced malignancy (Moore, 1980; Windsor & Hill 1988a, 1988b). Despite such assertions very little data has been published in the literature documenting the progress of cachexia in patients with pancreatic cancer. What little data there is, has looked at patients with a “snapshot” view rather than in a longitudinal manner (De Wys et al 1986). Chapter 3 documents the progress of cachexia in a group of untreated patients from the time of their diagnosis until a time point close to death. This study revealed that patients had lost almost one eighth of their body weight by the time of diagnosis and that by the time of death patients had lost approximately one quarter of their pre-illness stable weight. The pattern of weight loss appeared to be linear although some variation was observed between individual subjects. Anthropometric measurements confirmed marked depletion of muscle protein and subcutaneous fat reserves and showed that when compared to healthy reference values the majority of patients would be classified as severely malnourished. By the time of death protein depletion was so marked that, based on the work of Haydock and Hill (1986), patients were at serious risk of respiratory muscle embarrassment. This study emphasised the importance of cancer cachexia as a therapeutic target and that any therapeutic intervention should be instituted as early as possible since cachexia is already established in the majority of patients by the time of diagnosis.

At a very simple level body weight can be considered to reflect a balance between energy intake and energy expenditure. Chapter 4 attempted to address the question of energy balance in weight losing patients with pancreatic cancer and to relate this to the presence or absence of the APPR. Errors are large in the measurement of
energy intakes and total energy expenditure, despite this it was evident that a substantial energy deficit existed in the majority of patients. The major component of this energy deficit was accounted for by reduced nutritional intake consequent predominantly on anorexia. Elevated resting energy expenditure accounted for approximately one third of the total energy deficit although this varied widely between individuals. When patients were subdivided on the basis of whether they had an acute phase protein response, patients with an APPR were found to have significantly greater energy deficits due both to reduced nutritional intake and due to hypermetabolism. The association between the acute phase response and hypermetabolism has been previously described (Falconer et al 1994), however, the association between the APPR and anorexia has not. Anorexia has been linked to pro-inflammatory cytokines in particular TNF (Sherry et al 1989). Since TNF is known to be produced in tandem with IL-6 and both of these cytokines are stimulants of the hepatic APPR, the relationship between the APPR and anorexia is perhaps not surprising. One observation which was surprising (assuming a common stimulus for anorexia, hypermetabolism and the APPR) was the degree of variation between individuals with a similar level of APPR with respect to energy deficits due to both anorexia and hypermetabolism. The basis of this variation is not clear. Some of the observed variation would undoubtedly be explained by the errors in measurement of energy deficits. Part of the variation may also be due to inter-individual variations in both the production of and sensitivity to cytokines. Polymorphisms are known to exist in the TNF genes of healthy subjects (Wilson et al 1997) and it is also possible that genetic or other factors may influence the sensitivity of effector organs to cytokines. The clinical significance of the variation in the nature of energy deficits in individuals is two-fold. Firstly, it suggests that more detailed assessment of patients may facilitate the selection of an appropriate therapeutic strategy tailored to the individual’s needs. Secondly, it lends weight to the concept of using dual modality therapies which attempt to address both sides of the energy balance equation by providing adequate energy and also attenuating the metabolic abnormalities which may impair the utilisation of nutrients.
This thesis has attempted to address certain aspects of the mechanisms that lead to weight loss in cancer-bearing patients. In particular the role of the cytokine-acute phase protein response axis has been studied in depth in patients with cancer cachexia. Previous work has suggested that production of pro-inflammatory cytokines by peripheral blood mononuclear cells (PBMC), such as IL-6 and TNF, is elevated in weight-losing patients with pancreatic cancer (Falconer et al, 1994). In that study elevation of cytokine production by PBMC was most marked in patients with an ongoing APPR. There is now considerable evidence that lymphocyte-mediated regulatory processes may act to suppress pro-inflammatory cytokine production (Essner et al, 1989; Lee, et al, 1990; Fiorentino et al, 1991). This raises the important question of whether elevated production of cytokines by PBMC is refractory to suppression by lymphocyte-derived cytokines and whether using such a strategy might be of value as a means of attenuating the acute phase response. Chapter 5 attempted to address these questions using an acute and chronic disease model of activation of the APPR. This showed that production of IL-6 and TNF by PBMC from cancer patients and patients with acute illness are broadly susceptible to suppression using IL-4 in a similar manner to controls. Furthermore addition of IL-4 to PBMC cultures reduced the ability of their supernatants to stimulate acute phase protein production. IL-2 had little effect on PBMC cytokine production by any group but did reduce the ability of the PBMC supernatants to stimulate an acute phase response, the mechanism of this action is uncertain since IL-2 had no direct effect on hepatocytes. T-lymphocyte derived cytokines appear to be able to influence the production of pro-inflammatory cytokines by PBMC and to down-regulate the APPR in vitro. Translating these in vitro observations to a useful clinical strategy is difficult in view of the fact that the hepatotoxicity of IL-4 precludes its use in man and because IL-2 stimulates TNF and IFN-γ release in addition to its more immunosuppressive effects (Wigmore et al; 1995). Indeed in one study administration of recombinant IL-2 to patients with colorectal cancer resulted in augmentation of the acute phase and inflammatory responses to surgery (Deehan et al, 1994). It has been
proposed that improving T-lymphocyte function by more subtle means, such as by glutamine supplementation, might provide a mechanism of modifying the APPR without causing stimulation of another inflammatory pathway, however this theory remains unproven.

In Chapter 6 the production of cytokines (IL-8 and IL-6) capable of stimulating the APPR is described by human pancreatic cancer cell lines. Cytokine production has been described in a variety of animal models and by a broad range of cell lines isolated from human malignancies. The production of IL-8 by human pancreatic cancer cell lines represents a novel observation. IL-6 is the major stimulant of the hepatic acute phase protein response in man. In Chapter 5 the stimulant effect of both recombinant and human pancreatic cancer cell-derived IL-8 on human hepatic APP production is described. IL-8 has not previously been reported to be a stimulant of the human APPR. Although elevated concentrations of IL-8 and IL-6 were detected in the sera of approximately 45% of patients with pancreatic cancer and serum IL-8 correlated with serum C-reactive protein (Chapter 7), there is no direct evidence that IL-8 has an effect on the APPR in man or that human pancreatic tumours in vivo elaborate such pro-inflammatory cytokines. Large differences clearly exist in the ability of human tumours to generate an inflammatory and acute phase response in the host. The basis by which a human tumour results in immunostimulation is not clear. It has often been argued that a vigorous immune response to a tumour represents a desirable reaction (Deehan et al; 1994). This might be true were it not for the tremendous degree of redundancy in the immune system and the stereotypical nature of the immune response that results in not only desirable activation of NK cells and cytotoxic T-lymphocytes but also results in activation of PBMC. The mechanism of this activation might be a potential therapeutic target as it does not appear to offer a clear benefit and tumours associated with a systemic inflammatory response generally have a worse prognosis than tumours of a similar type that do not excite such a response. Such intervention would clearly have to preserve the cell mediated immune response to the tumour while suppressing the non-
specific element. Similarly the extent to which tumours elaborate cytokines in vivo is an interesting area for future research using methods such as in situ hybridisation to determine whether such cytokines are produced in human pancreatic tumours.

Previous studies had established that an association existed between the APPR and the presence of elevated resting expenditure. Chapter 8 described a short term study which was designed to establish whether therapeutic attenuation of the APPR would be associated with reduction in resting energy expenditure. In this study administration of the non-steroidal anti-inflammatory drug ibuprofen resulted in reduction of serum CRP in the majority of patients and this was associated with a significant reduction of resting energy expenditure. These effects were not observed in patients receiving placebo suggesting that this was a bona fide consequence of ibuprofen administration. Whether the APPR is such an energy dependent pathway that it drives hypermetabolism in patients with cancer cachexia or whether it is simply a marker for activation of a whole range of metabolic and inflammatory pathways is not clear. Previous studies have demonstrated that ibuprofen suppresses the metabolic response to burn injury however whether this effect is mediated through a central hypothalamic pathway or through suppression of a metabolic pathway such as the APPR is not known (Wallace et al, 1992). Clearly it is important to establish the precise nature of the relationship between the APPR and elevation of resting energy expenditure. The range of non-steroidal anti-inflammatory drugs available has now increased dramatically and with the development of this class of drug has come the realisation that considerable variation in specificity of action exists within members of this group. Certain drugs e.g. tenidap (Wyllie et al 1996) have been shown to have a marked effect in reducing the APPR while others are less effective, this might provide a starting point for future research into the relationship between the APPR and resting energy expenditure.

In order to investigate the longer term effects of ibuprofen administration on nutritional indices of patients with pancreatic cancer a further clinical trial was
undertaken (Chapter 9). This trial also attempted to address a second question of whether the use of a drug with the potential to alter the metabolic profile of a patient in combination with an appetite stimulant might offer greater advantage than provision of an appetite stimulant alone. It could be argued that a trial of ibuprofen versus no treatment might have been preferable to the concurrent use of megestrol acetate. The reason megestrol acetate was chosen was firstly, to evaluate the effect of ibuprofen in a situation where both nutritional intake and appetite had been improved. Secondly, recruitment to a trial which has two treatment arms is more easy than to a trial which has a non-treatment arm and is ethically more acceptable to both health care professionals and patients. This trial showed that subjective scores of appetite and nutritional intake were improved in both patients receiving megestrol acetate and ibuprofen and in those receiving megestrol acetate and placebo and this was reflected in a similar increase in recorded energy intakes in the two groups. Despite these observations, only patients receiving megestrol acetate and ibuprofen experienced reduction of rate of weight loss or weight gain whereas patients receiving placebo continued to lose weight. The difference in weight change between the two groups was statistically significant and was associated with similar changes in anthropometric values suggesting that weight gain was not simply a reflection of fluid retention but had been associated with gain in lean tissue and subcutaneous fat. This data supports the concept of using a metabolism modifying drug to attempt to attenuate the catabolic pathways which may prevent the accretion of lean tissues in patients with cancer cachexia.

One disappointing aspect of this study was that despite producing a significant attenuation of cachexia in the group of patients receiving megestrol acetate this did not lead to a significant survival advantage. The probable reason for this is two fold. Firstly, the patients who were entered into the study were at the end stage of the natural history of their disease with 85% of the study group having clinical evidence of metastatic disease. The life expectancy of the patients was therefore extremely short and any
survival difference could be expected to be very small. Had patients with earlier stage disease been entered then it is possible that the survival difference may have been more evident. Secondly, the drugs used in the study did not have potential to alter significantly the biological behaviour of the cancers and so could not be expected to alter non-cachexia-related morbidity and mortality.

A gratifying aspect of the study was that quality of life indices were improved significantly in patients receiving ibuprofen compared with those receiving placebo. In trials of palliative agents quality of life assessment is important and the improvements in the present study appeared to be principally related to rather non-specific factors such as “improvement in sense of well-being” in addition fatigue scores were lower and patients were more able to maintain an independent existence without reliance on carers. Future assessment of non-steroidal anti-inflammatory drugs could develop in a number of directions. NSAIDs such as tenidap exert a greater suppression of the APPR than previous members of the same class of drug and as such may be more effective in the management of cancer cachexia. Since the mechanism of action of NSAIDs in cancer cachexia is by no means clear it is possible that NSAIDs in combination with other immunomodulatory agents such as EPA may have a greater effect than in isolation.

In Chapter 10, a study was undertaken to evaluate the potential of fish oil derived polyunsaturated fatty acids to modify the progress of cachexia in patients with pancreatic cancer. The rationale for this study was that fish oil had been associated with attenuation of cachexia in animal models of cancer cachexia, anti-tumour effects in vitro and in vivo and had been shown to down-regulate production of cytokines known to be associated with stimulation of cancer cachexia and anorexia such as TNF and IL-1. Oral administration of fish oil containing 18% eicosapentaenoic acid was associated with attenuation of weight loss in the majority of patients with pancreatic cancer. Prior to supplementation all patients had been losing weight but following 3 months
supplementation 14 of 18 had either become weight stable or had gained a small amount of weight. In addition the remaining 4 patients who continued to lose weight did so at a greatly reduced rate.

The mechanism of this effect of fish oil supplementation was not clear, measurement of serum CRP demonstrated a significant but transient reduction in APPR. Similarly a direct correlation was observed between reduction of serum CRP and reduction of resting energy expenditure. This is the first trial of its type to demonstrate a consistent attenuation of cachexia in patients with cancer. Patients who had received a related fatty acid GLA in a trial with similar aims and endpoints had continued to lose weight suggesting that the effects of fish oil were not simply a placebo effect. On the basis of this study further research was undertaken to investigate the effects of fish-oil derived polyunsaturated fatty acids on cancer cachexia.

A second study was undertaken to attempt to ascertain whether EPA was the active component of fish oil responsible for the anti cachectic effects described above (Chapter 11). In this trial 95% pure EPA was given to patients in the form of the encapsulated free acid. The results of this study were very similar to those observed in the previous trial (Chapter 10) in that attenuation of weight loss was observed in the majority of patients. The changes in rates of weight loss were greater in responders than in the fish oil study such that weight gains were greater and more sustained. The proportion of responders to non-responders was similar to the Fish oil study and there was no clear advantage of supplementation with EPA over Fish oil. The dose of EPA in the high purity EPA trial was 6g per day compared with 2.12g per day in the fish oil trial. This represents a relatively small concentration range and it is possible that higher concentrations of EPA may be more effective particularly in relation to anti-tumour effects.
The mechanism of action of EPA in attenuating human cancer cachexia is not certain. The in vitro studies described in Chapter 11 demonstrated that oral EPA supplementation was associated with reduction of pro-inflammatory cytokine production by human PBMC and that this was associated with reduced potential of PBMC supernatants to stimulate an APPR. The effects of EPA on the APPR in vivo were not however predictable and no consistent reduction of serum CRP concentration was observed. This may be the consequence of looking at snapshots of a continuum but also may indicate that some other mechanism is involved. Reduction in cytokine production has the potential to reduce cytokine mediated suppression of appetite and the clinical trial of high purity EPA did demonstrate a small increase in nutritional intake in a number of subjects. EPA might have had an anti-tumour effect. Accurate assessment of tumour size in patients with pancreatic cancer is notoriously difficult even with modern high resolution imaging technology. No formal assessment of tumour size was made in any of the clinical trials described in this thesis. Survival is a reasonably good indicator of tumour response in patients with advanced pancreatic cancer because prognosis is universally poor in this patient group. In both of the trials of EPA containing compounds median survival duration was 6 months from the start of treatment. This survival duration is as good as the best chemotherapy trials and as such may represent a small anti-tumour effect. Tumours were assessed by serial CT scanning in a small number of patients receiving high purity EPA and the best response in this group was of static disease over 6 months periods.

It is possible that EPA may have exerted its anti-cachectic effect through a different pathway such as via suppression of a direct tumour-derived cachectic factor. This cachectic factor which was first identified in the murine MAC 16 colon adenocarcinoma model of cachexia has recently been identified in the urine of weight losing patients with pancreatic cancer (Todorov et al 1996). EPA has been shown to have an anticachectic effect in the MAC 16 colon cancer model and it is possible that this is mediated through suppression of one or more cachectic factors (Tisdale 1996).
The effect of EPA on the expression of this cachectic factor is the subject of current research.

The effectiveness of EPA containing compounds in the two phase I trials described in this thesis justify further evaluation of EPA in the context of a controlled trial. The design of such a trial would be of critical importance as unrealistic outcome measures and inadequate sample size have been the ruination of many potentially effective drugs in cancer research. Such a trial would be expensive and identifying sponsorship for such a study would be difficult given the general unpopularity of palliative strategies.

In this thesis encouraging responses have been obtained to novel therapeutic strategies designed to attenuate cachexia. Improved responses are likely once more research has been undertaken into a number of crucial areas. Firstly the nature of the relationship between the acute phase response and resting energy expenditure and anorexia. Secondly the method of coupling or signalling between liver protein pathways and skeletal muscle. Thirdly the potential interaction between cytokines and the ubiquitin-proteosome and leptin-hypothalamic pathways may be a subject for future research. Cytokines almost certainly have a role in many of these areas however the precise interactions are not understood and the linkage between the cytokines and counterregulatory hormones, direct cachectic factors and neural pathways are not understood. Basic science research in this field is particularly difficult since the levels of possible interaction are so great that modelling can become very complex and interpretation can be similarly prone to error or misinterpretation.

Therapeutic strategies directed against cachexia in patients with cancer have the potential to influence several aspects of the natural history of cancer. Improving nutritional status may prolong life by preventing protein depletion which may ultimately lead to respiratory muscle embarrassment and hypostatic pneumonia. In this
thesis no significant survival advantage was demonstrated in patients with advanced pancreatic cancer receiving ibuprofen and megestrol acetate compared with those receiving placebo and megestrol acetate. As palliative trials of nutritional therapy gain credibility it will be possible to enter patients with earlier stage disease (for whom there is still no effective cytotoxic therapy) and overcoming the lag effect of being at the end of the natural survival curve may produce a genuine survival advantage. In addition to prolonging life there exists the potential to improve the quality of life by prevention of lean tissue depletion. This can lead to reduction of physical and psychological morbidity and allow patients to maintain an independent existence. One of the long term goals of nutritional therapy in patients with malignant tumours is to enable the host to tolerate cytotoxic therapy better, thus, increasing the potential of treatment to modify the growth and behaviour of the tumour without accelerating decline of the host. The use of TPN has not improved the response of patients with cancer to chemotherapy, radiotherapy or surgery compared with patients not receiving nutritional support. Since TPN represents a non-targeted intervention this is not surprising and therapies with the potential to influence metabolic and inflammatory pathways may be more successful. The role of compounds such as EPA in combination with cytotoxic chemotherapy is the subject of an ongoing clinical trial in patients with recurrent small cell lung cancer and may be evaluated in gastrointestinal cancers in the future.

This thesis has addressed some of the mechanisms of cancer cachexia and has also demonstrated that successful therapeutic intervention in this disease is possible. One of the valuable aspects of both basic science and clinical research is that it frequently raises more questions than it answers. This thesis is no exception and there are a number of clear opportunities for further investigation. Raising the profile of cancer cachexia and its human and economic importance remains a difficult task but one which is a necessary prelude to securing major funding for research in this field.


AKIRA S, KISHIMOTO T. IL-6 and NFKB-IL-6 in acute phase response and viral infection.


HUDSON EA, TISDALE MJ. 1994 Comparison of the effectiveness of eicosapentaenoic acid administered as either the free acid or ethyl ester as an anticachectic and antitumour agent. Prostaglandins Leukotrienes & Essential Fatty Acids. 51(2):141-5.


KOJ A. 1985. The acute phase response to injury and infection. (Elsevier, Amsterdam) 10; 139.


POLI V, OLIVIERO S, MORRONE G, CORTESE R. 1989. Characterisation of an IL-6 responsive element (IL6RE) present on liver-specific genes and identification of the
cognate IL-6-dependent DNA-binding protein (IL-6DBP). Ann NY Acad Sci 557: 297-309.


APPENDIX 1

List of presentations relating to submitted work

BRITISH SOCIETY OF GASTROENTEROLOGY 1994, 1995

ω-3 and ω-6 fatty acids upregulate IL-6 stimulated hepatocyte acute phase protein production in vitro

Human pancreatic cancer cell lines constitutively produce IL-8 and IL-6.

Titres of serum IL-8 correlate with the presence of an acute phase response in patients with pancreatic cancer.


Pancreatic cancer cells stimulate C-reactive protein production in isolated hepatocytes via IL-6 or IL-8

Humoral immunity to endotoxin is preserved in patients with progressive carcinoma of the pancreas.

Cytokine regulation of human pancreatic cancer cell-derived IL-8 production.

Eicosapentaenoic acid, cytokines and the acute phase response in pancreatic cancer.


EUROPEAN SOCIETY FOR PARENTERAL AND ENTERAL NUTRITION 1994, 1995

Ibuprofen reduces resting energy expenditure and C-reactive protein production in weight-losing patients with cancer
Arrest of weight loss in cachectic pancreatic cancer patients receiving fish oil supplementation.

The nutritional status of patients with unresectable cancer of the pancreas.

Production and regulation of pro-inflammatory cytokines by human pancreatic cancer cell lines.

Pancreatic cancer cell-derived IL-8 stimulates hepatic acute phase protein production in vitro and IL-8 is elevated in the sera of weight losing patients in vivo.

Ibuprofen vs placebo in the treatment of pancreatic cancer cachexia.

The acute phase response and survival duration in pancreatic cancer

The effect of eicosapentaenoic acid supplementation on PBMC cytokine production and the acute phase response in pancreatic cancer.

Influence of oral fish oil supplementation on weight loss in pancreatic cancer patients.

NUTRITION SOCIETY CMNSG 1995, 1996
Plasma and erythrocyte membrane fatty acid composition in pancreatic cancer patients receiving dietary fish oil

Attenuation of weight loss in patients with pancreatic cancer receiving fish oil.

Randomised double blind placebo controlled trial of megestrol acetate and ibuprofen in patients with advanced pancreatic cancer.

The acute phase response and energy balance in pancreatic cancer cachexia

EUROPEAN INTENSIVE CARE SOCIETY GLASGOW

299

ASSOCIATION OF SURGEONS OF GREAT BRITAIN AND IRELAND 1995, 1996


OTHERS

The acute phase response and survival duration in pancreatic cancer
APPENDIX 2

List of publications relating to submitted work

Fatty acids for treating pancreatic cancer

Ibuprofen reduces resting energy and acute phase protein production in patients with pancreatic cancer.

The acute phase response and survival duration in pancreatic cancer.

The effect of polyunsaturated fatty acids on the progress of cachexia in patients with pancreatic cancer.

Weight loss and colorectal cancer
S.J. Wigmore & K.C.H. Fearon 1995 Colonews 4(4) 1-4

Modulation of the cytokine and acute-phase response to major surgery by recombinant interleukin-2.

Changes in nutritional status associated with unresectable pancreatic cancer.

Modulation of human hepatocyte acute phase protein production in vitro by n-3 and n-6 polyunsaturated fatty acids.

The contribution of anorexia and hypermetabolism to energy deficit in patients with unresectable pancreatic cancer

Down-regulation of the acute phase response in patients with pancreatic cancer cachexia receiving oral eicosapentaenoic acid is mediated via suppression of interleukin-6.

Induction of cachexia in mice by a product isolated from the urine of cachectic cancer patients.

Interleukin-8 can mediate acute-phase protein production by isolated human hepatocytes.