AN INVESTIGATION OF THE RELATIONSHIP BETWEEN THE SYSTEMIC AND LOCAL INFLAMMATORY RESPONSES AND SURVIVAL IN PATIENTS WITH PRIMARY OPERABLE COLORECTAL CANCER

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DECLARATION

I declare that I have solely carried out the work presented in this thesis, except where indicated below.

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DEDICATION

To my wife

I dedicate this work for her help, encouragement and support during the writing of the thesis, and to my sons Awas and Harith and my two little girls Sara and Reem.

Summary

Colorectal cancer remains the second commonest cause of cancer death in Western Europe and North America. Overall survival is poor. Even in those patients who undergo potentially curative resection more than one third will die within 5 years.

It has long been recognised that disease progression in cancer patients is not solely determined by the characteristics of the tumour but also by the host response. One aspect of the host response, which has recently generated interest, is the non-specific systemic inflammatory response, associated with primary operable colorectal cancer. There is evidence that the systemic inflammatory response (as evidenced by C-reactive protein) predicts recurrence, and overall and cancer specific survival, independent of stage, in patients who have undergone curative resection for colorectal cancer.

Another aspect of the host response is tumour lymphocytic infiltration, there is increasing evidence that both local and systemic inflammatory responses play an important role in the progression of a variety of common solid tumours. In patients with colorectal cancer, there is good evidence that, on simple staining of tumour sections, the presence of a pronounced lymphocytic infiltration within the tumour is associated with improved survival.

In the first research chapter of this thesis we assessed the value of combining Dukes stage and C-reactive protein to form a new prognostic score in patients undergoing apparently curative resection for colorectal cancer. The results suggest that this simple prognostic score, which reflects both the contribution of the tumour and the host response, appears to differentiate between low risk and high risk Dukes B and C

tumours and may therefore be useful in selecting appropriate patients for adjuvant chemotherapy.

In the second research chapter we examined the relationships between the systemic inflammatory response, tumour lymphocytic infiltration, tumour proliferative activity and cancer survival in patients with Dukes stage B and C colorectal cancer undergoing potentially curative resection. A poor tumour CD4+ and CD8+ T-lymphocyte infiltrate, an increased Ki-67 labelling index were associated with poorer cancer specific survival. Poor tumour CD4+ T-lymphocyte infiltrates, increased Ki-67 labelling index and increased tumour diameter were associated with an elevated circulating C-reactive protein.

Multivariate survival analysis showed that both local (tumour CD8+ T-lymphocyte infiltrate) and systemic (C-reactive protein) inflammatory responses, but not tumour proliferation (Ki-67 labelling index), were significantly associated with cancer specific survival. That tumour proliferation is a relatively poor predictor of survival compared with the systemic inflammatory responses, possibly relates to the fact that tumour dissemination, rather than tumour proliferation, is the primary determinant of survival in patients with colorectal cancer.

These results would suggest that local and systemic inflammatory responses are linked to tumour proliferation in patients with colorectal cancer and these responses are in turn linked to cancer specific survival, independent of tumour stage. This means that, both local and systemic inflammatory responses are intrinsically linked to cancer specific survival in patients who have undergone apparently curative surgery for colorectal

cancer. Furthermore, these results would suggest that the presence of a systemic inflammatory response reflects increased tumour bulk and compromised cell mediated immunity.

1.0 COLORECTAL CANCER

1.1 Epidemiology of Colorectal Cancer

Colorectal cancer is the third most common malignancy in the developed world, (Coleman, 1999). Worldwide, approximately 780,000 new cases were diagnosed in 1990, which represents 9.4% of all incident cancer in men and 10.1% in women (Boyle, 2000).

Colorectal cancer is not uniformly distributed among all populations. The highest incidences are in Western Europe and North America, whereas intermediate rates prevail in Eastern Europe. The lowest rates are seen in Asia, Africa and South America. However, there is some evidence that the incidence of the disease in Africa is now increasing, probably as a result of improving lifestyle (Iliyasu et al, 1997).

In the UK, colorectal cancer is the third most common form of cancer after lung and breast cancer. More than 35,000 new cases are diagnosed annually. It is now second only to lung cancer as a cause of cancer death in the UK, with over 16,000 deaths each year, which accounts for 11% of the UK cancer related mortality (Cancer Research UK, 2004, www.cancerresearchuk.org).

Colorectal cancer affects almost equal proportion of men and women, most commonly between the ages of 60 and 80 years, but when the incidences of cancer of colon and rectum are examined separately, certain differences in sex incidence emerge. The ratio between males and females in the UK for cancer of the colon is approximately 2:3 and for cancer of the rectum is approximately 8:7 (Keighley and Williams, 1999).

Most authors seem to agree about the distribution of tumours within the colon, Thus, approximately 50% of all colon cancers are in sigmoid, 25% are in right colon (caecum and ascending colon), and the remaining 25% are in the transverse colon, splenic flexure, descending colon and hepatic flexure, in descending order of frequency (McDermott et al, 1981).

The distribution of cancer within the rectum is difficult to determine because authors who have studied this subject have often used different measurements when dividing the rectum into its upper, middle and lower segments. The consensus, however, seems to be that, apart from minor variations, rectal cancers are equally distributed between the three segments of the rectum (Keighley and Williams, 1999).

1.2 Actiology

The development of colorectal cancer represent the culmination of a complex process in which both hereditary and environmental factors, acting either alone or in combination, cause changes in the regulatory genes and cellular dysfunction. Most of the colorectal cancer (90- 95%) arise from sporadic adenomas (polyps) and most of the remaining are accounted for by several hereditary cancer syndromes. Despite advances in understanding the biology and natural history of colorectal cancer survival has not significantly improved in recent years.

1.2.1 Genetic Factors

Adenomatous polyps are widely believed to be the precursors of most of the colorectal adenocarcinomas and this concept is supported by epidemiological, genetic and pathological studies. Patients with adenomatous polyps have a threefold higher risk of colon cancer over the general population and the risk increases to six-fold if the polyps are multiple. The evolution of a carcinoma from an adenoma is generally believed to be a multi-step process of tumorigenesis called the, "adenoma- carcinoma sequence". This concept, postulated in the mid-seventies is now widely accepted and has been supported by molecular genetics studies (Tomlinson et al, 1997).

However, the genetics events that take place in the development of sporadic colorectal cancer are also being explained. Mutations of the adenomatous polyposis coli (*APC*) gene, which is probably involved in cell-cell adhesion, are thought to occur early as they are found in 60% of all adenomas and carcinomas (Powell et al, 1992). K-*ras* mutations, which probably stimulate cell growth by activating growth factor signal transduction, similarly occur in both adenomas and carcinomas. However, as they are more common

in large adenomas than small adenomas they are thought to represent a later event (Vogelstein et al, 1988; Scott et al, 1993). The deleted in colorectal cancer (*DCC*) gene is a tumour-suppressor gene that may be responsible for cell-matrix interactions (Fearon et al, 1990), and its deletion may be important in further progression towards the malignant state. Mutation of the P53 gene is common in invasive colonic cancers but rare in adenomas and is therefore deemed to be a late event which accompanies the development of the invasive phenotype (Kikuchi-Yanoshita et al, 1992). The P53 gene is a tumour suppressor gene that appears to be the most important determinant of malignancy in colorectal neoplasia. It is located on the short arm of chromosome 17 and is frequently lost in colorectal malignancy. It is considered to be a transcription factor because it activates other genes and promotes their expression. These genes are involved in growth inhibition and loss of the P53 gene may therefore lead to unregulated cellular growth. Mutations of P53 are found in more than 50% of all human cancers and more than 75% of colorectal adenocarcinomas, making it one of the most important factors in human carcinogenesis (Vogelstein et al, 1988; Greenblatt et al, 1994). The varied functions of P53 including control of the cell cycle, DNA repair and the induction of programmes cell death (apoptosis) to prevent proliferation of cells with deleterious mutations (Bukholm et al, 2000). Mutant P53 protein has a half-life of approximately 24 hours compared to wild-type P53 protein which has a half-life of 20 minutes. Studies have shown that mutation in P53 usually result in the accumulation of P53 proteins (overexpression), which in turn result in increased proliferation, loss of apoptotic function, and chromosomal instability (Buglioni et al, 1999; Bukholm et al, 2000).

The sequence of events described above is shown in Figure 1.1 (Smith et al, 2002), but it must be stressed that this merely illustrates one possible multistep process; indeed, there is now good evidence that K-*ras* and P53 mutations very rarely occur in the same tumour, suggesting alternative pathways to carcinogensis (Smith et al, 2002).

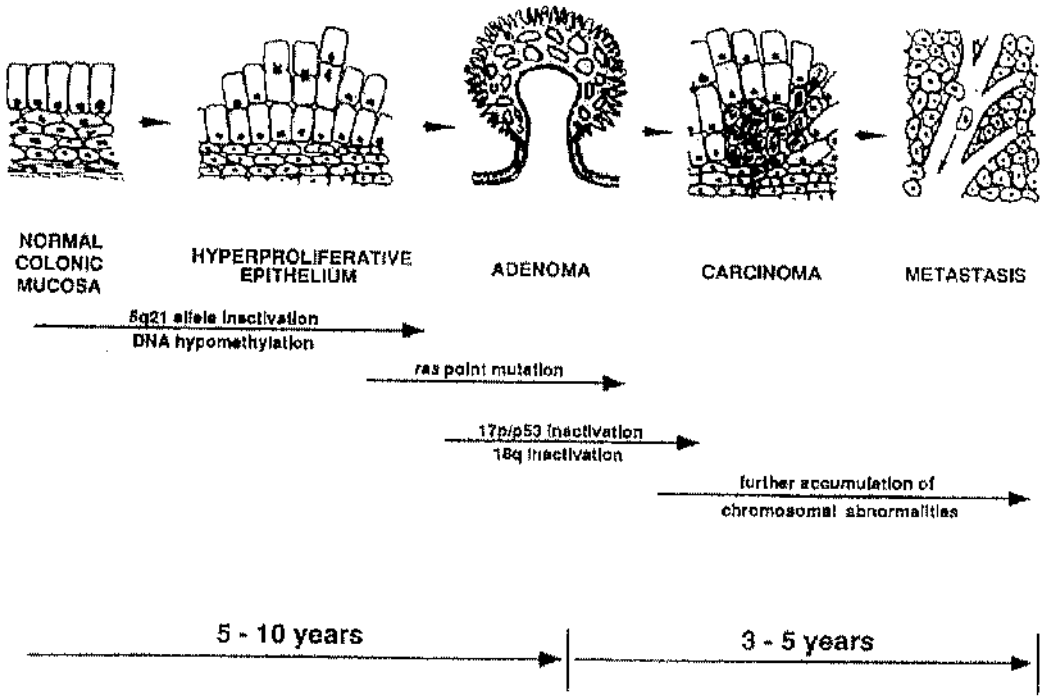


Figure 1.1 Adenoma- carcinoma sequences (adapted from Smith et al, 2002).

1.2.2 Inherited Bowel Cancer

Hereditary non-polyposis colorectal cancer

HNPCC is responsible for about 2% of colorectal cancer and is the commonest of the main inherited bowel cancer syndromes. HNPCC was previously known as Lynch syndrome and is inherited in an autosomal dominant fashion.

HNPCC is characterised by early onset of colorectal tumour, the average age at diagnosis being 45 years compared with approximately 65 years in the general population. These tumours have certain distinguishing pathological features. There is a predilection for the proximal colon, and tumours are also frequently multiple (Synchronous and metachronous). They tend to be mucinous, poorly differentiated and of 'signet-ring' appearance, with marked infiltration by lymphocytes and lymphoid aggregation at their margins.

HNPCC is due to germline mutations in mismatch repair (MMR) genes, whose role is to correct errors in base-pair matching during replication of DNA or to initiate apoptosis when DNA damage is beyond repair. The MMR genes are tumour suppressor genes. Defective MMR genes result in the accumulation of mutations in a host of other genes, leading to tumour formation.

Defective MMR genes also results in micro satellite instability (MSI), a hallmark of tumour in HNPCC (Frayling, 1999). Micro satellites are regions where a short DNA sequence (up to five nucleotides) is repeated. There are large numbers of such sequence in the human genome, the majority in non-coding DNA. Base-pair mismatches occurring during DNA replication are normally repaired by the MMR proteins. In the

tumour with deficiency of these proteins this mechanism fails and micro satellites become instable (micro satellite instability).

Familial Adenomatous polyposis (FAP)

Familial adenomatous polyposis (FAP) is an autosomal dominant, inherited disorder, less common than HNPCC, with 100% risk of developing colorectal cancer (Talbot, 2000). It is characterized by the development of hundreds of adenomas during the second and third decades of life. These occur predominantly in the large bowel, but adenomas may also occur in the upper gastrointestinal tract. Genetic studies have shown that FAP caused by mutation in the tumour-suppressor adenomatous polyposis coli (APC) gene on chromosome 5. The sub site distribution of colorectal carcinoma in patients with familial adenomatous polyposis (FAP) is similar to that in patients with sporadic colorectal cancer; in both, left-sided cancers are more frequent than right-sided cancer (Bufill, 1995).

1.2.3 Environmental Factors

Diet

Meat, Animal protein and Fat

Evidence from epidemiological studies seems to show consistently that there is a well-established relationship between intake of certain dietary constituents, especially meat, and colorectal cancer.

Armstrong and Doll (1975) first described the association between incidence and mortality rates of colorectal cancers in 32 countries with per capita consumption of meat, animal protein, and total fat consumption. The correlation coefficient for meat and

animal protein consumption remained higher than 0.7 after adjusting for other variables. The correlation was stronger for colon rather than for rectal cancer.

More recently, Sinha and co-workers (1999) have shown that well done, grilled red meat increases the risk of colorectal cancer. This consistent with the hypothesis that carcinogenic compounds formed by high temperature cooking techniques, such as heterocyclic amines and polycyclic aromatic hydrocarbons, may contribute to the risk of developing cancer.

Population studies and the great majority of earlier case-control studies suggested that colorectal cancer risk increased with an increased intake of dietary fat (Miller et al, 1983; Graham et al, 1988). Several more recent case-control studies have also shown that there was a positive correlation between dietary fat intake and colorectal cancer (Panel on Food, Nutrition and the Prevention of Cancer; 1997; Lipkin et al, 1999). Since then, a substantial amount of progress has been made in understanding the relationship between dietary fat and development of colorectal cancer in humans. In 1990, Willett and co-workers published the results from the US nurses health study involving follow up of almost 89, 000 women aged 34-59 years who were without cancer or inflammatory bowel disease at recruitment. After adjustment for total energy intake, consumption of animal fat was found to be associated with increased risk of colorectal cancer.

On the other hand, several large prospective studies have been reported in Europe and the United States up to 2001. None of these studies found a clear, positive association between fat and colorectal cancer (Slattery et al, 1997; Terry et al, 2001; Jarvinen et al,

2001). Indeed, Giovannucci and co-workers (1994) noted that the increased risk associated with animal fat intake in the American nurses disappeared when red meat intake was taken into the account.

Furthermore, in a combined analysis of 13 case control studies, Howe and co-workers (1997) showed no measurable positive association between either total fat intake or intake of saturated fat and colon cancer after adjustment for total calorie intake. Therefore, while animal studies have suggested an aetiological role for high fat intake in colorectal carcinogenesis, such evidence is very hard to extrapolate to humans (Kono, 2001).

Fibre

Burkitt's hypothesis (1971), that a lack of dietary fibre is associated with the higher incidence rates of colorectal cancer in western countries, generated a vast amount of epidemiological research. Two meta-analyses of 13 and 20 case control studies appeared to show that high intake of fibre-rich food reduced the risk of colorectal cancer by one-half (McKeown-Eyssen, 1985; Howe, 1992). However, in a prospective cohort study, only fibre from fruit was associated with an appreciable reduction in risk, but this relation was not statistically independent from meat intake (Willett, 1990)

More recently, Fuchs and co-workers (1999) analysed outcome in the nurses' study with regard to fibre intake. During a 16-year follow-up period, 787 cases of colorectal cancer were documented. Furthermore, adenomas were detected in 1012 patients out of 27,530 participants who underwent endoscopies during the follow-up period. After adjustment for age, established risk factors, and total energy intake, they found no association

between the intake of dietary fibre and the risk of colorectal cancer. The results of the more recent larger studies therefore, do not support the existence of an important protective effect of dietary fibre against colorectal cancer or adenoma.

Minor dietary constituents

A number of dietary constituents have been found to inhibit carcinogenesis, including selenium, vitamin C, E, retinoids, carotenoids (ß-carotene, lycopene, lutein, ß-cryptoxanthin, and α-carotene) and plant sterols. It is worth noting that, diets low in Vitamin C and carotenoids also tend to be low in fibre (Pritchard, 1996). Carotenoids, long recognized for their antioxidant properties, are of increasing interest in relation to cancer because of their effect on regulation of cell growth (Rock, 1997). Recent studies by Slattery and co-workers (2000) have suggested that there may be an association between the use of these supplements (lutein and α-carotene) and a low risk of developing colorectal cancer.

Tobacco and Alcohol

There is little evidence that colon cancer is associated with consumption of alcoholic beverages. Of 15 cohort studies published up to 1992, none showed significant alcohol-related increase in the risk of colon cancer. Rectal cancer, however, was positively associated with alcohol in number of cohort studies (McMichael and Giles, 1994).

Until recently there was little evidence that smoking was related to colorectal cancer (Sandler, 1988; Slattery, 1990; Kune, 1992). However, two reports using data from two large single-gender cohorts of health professionals concluded that for each gender the number of cigarettes smoked during the most recent 20 years was positively related to

the prevalence of small adenomas, whereas pack years of smoking more than 20 years earlier was linked with an increase in large adenomas. Smoking was related to risk of colorectal cancer after allowing for an induction period of at least 35 years (Giovannucci, 1994a; Giovannucci, 1994b; Giovannucci, 2001).

On the other hand Nyren and coworkers (1996) carried out a large cohort study, which involved approximately 135,000 male construction workers, with an average follow up 17.6 years. This study did not support a high risk of colon cancer in males who were long-term heavy smokers and provided only weak support for an association with rectal cancer.

1.2.4 Biological markers and colorectal cancer

Apoptosis

The normal gastrointestinal epithelium is characterized by rapid cell turnover at the level of the colonic crypt, replacing the surface epithelium approximately every six days. The highly proliferative compartment is the lower third of the crypt. As the cells move up the crypt, they mature, differentiate, lose their dividing ability and eventually die (apoptosis).

Apoptosis, or programmed cell death, plays an important role in many physiologic and pathologic processes (Thompson, 1995). Among others, an important function of apoptosis lies in the elimination of damaged cells. For example, cells with genetic damage caused by exposure to carcinogens may be deleted by undergoing apoptosis, thereby preventing their replication and the accumulation of clones of abnormal cells. There is increasing evidence to support the hypothesis that failure of apoptosis may be

an important factor in the evolution of colorectal cancer and its poor response to chemotherapy and radiation (Watson, 1995). Inhibition of apoptosis causes an imbalance in normal tissue homeostasis promoting cell growth and it also allows the survival of genetically damaged cells, both contributing to tumour development and progression (Hawkins et al, 1997).

Traditionally, an increase in cell proliferation, rather than a change in apoptosis, has been used to predict later tumour development and prognosis. While there is general agreement that the dysregulation of apoptosis contributes to malignant transformation, the potential predictive or prognostic value of the degree of apoptosis in colorectal cancer is controversial. Several studies have examined the prognostic value of the apoptotic index in colorectal cancer, producing conflicting results (Hawkins et al, 1997; Hashimoto et al, 1997; Kawasaki et al, 1998; Evertsson et al, 1999; Sinicrope et al, 1999; Tenjo et al, 2000; Schwandner et al, 2000; Paradiso et al, 2001). Schwandner and co-workers (2000) showed that the apoptotic index was not predictive of prognosis in a series of 160 cases of rectal cancer. In two studies, it was shown that a low apoptotic index in the tumour was associated with poor survival (Langlois et al, 1997; Kawasaki et al, 1998). Two reports showed that apoptotic indices were higher in tumours that were more highly differentiated and had not invaded or metastasised than in those that were poorly differentiated and invasive or metastasizing (Hashimoto et al, 1997; Sugamura et al, 1998).

The degree of apoptosis in colorectal tumours is not related to the degree of proliferation in most studies. The use of the apoptotic index in a tumour as a potential

prognostic marker or indicator for the choice of therapy, as suggested by some, is currently not supported by the available data.

Proliferation

Ki-67 protein is a large nuclear antigen expressed in highest concentrations in all stages of the cell cycle but not in resting cells. It has a very complex and specific localization pattern within the nucleus, one which changes during the cell cycle. It has been known for some time that Ki-67 protein is associated with the dense fibrillary component (DFC) of the nucleolus (Verheijen et al, 1989). Although there is a vast amount of information known about the structure, localization and regulation of Ki-67 protein, but there is little information about the function of this protein apart from the fact that it is vital for cell proliferation (Brown and Gatter, 2002).

The original antibody raised against Ki-67 protein could be used only on fresh or frozen tissue, since fixation greatly interferes with immunostaining pattern. This prototypic antibody was succeeded by other antibodies which could recognize Ki-67 protein in conventional fixed tissue sections, treated with microwave irradiation (Rose et al, 1994). The most widely used of these is monoclonal antibody MIB-1 (Molecular Immunology Borstel) (Cattoretti et al, 1992). There is no doubt that this antibody has been the single largest achievement in the investigation of Ki-67 protein's prognostic potential. Its arrival meant that retrospective studies using archived surgical histology material could be undertaken and large prospective studies became much easier to perform since there was no longer the need to collect fresh tissue. The immunohistochemical staining pattern of ki-67 antigen has been found to correlate well

with tumour proliferative activity in various human malignancies (Brown and Gatter, 2002).

It is well established that Ki-67 protein is of prognostic value for many types of malignant tumour as it represent tumour proliferative activity and for many tumour types, high proliferative activity has been shown to correlate with poor clinical outcome. For example, in breast cancer most studies showed a statistically significant correlation between high proliferative activity and clinical outcomes, both on univariate and multivariate analysis (Locker et al, 1992; Beck et al, 1995; Jacquemier et al, 1998; Chang et al, 1999). Other studies reveal an unclear and often contradictory pattern for certain tumours, e.g. cervix uteri, non-Hodgkin's lymphoma and prostate cancer (Brown and Gatter, 2002).

In colorectal cancer, the relation between proliferation marker and prognosis after resection are conflicting. For example, Brown and Gatter (2002) in their recent review of those studies, only three out of 12 studies reported a significant association with survival (Palmqvist et al, 1999; Saleh et al, 1999; Kimura et al, 2000). The results of the above studies which did show a correlation were also contradictory; for example, Palmqvist et al (1999) concluded that colorectal carcinomas with low Ki-67 protein expression at the invasive margin had a poor prognosis, whilst Saleh and co-workers (1999) and Kimura and co-workers (2000) showed that a high Ki-67 protein expression at the site of invasive margin had a worse prognosis. These apparently contradictory findings may be the result of the marked heterogeneity of Ki-67 protein expression in colorectal carcinomas (Ofner et al, 1996). However, many of these studies were based

on small numbers; some included both primary operable and advanced disease and most used semi-quantitative techniques for the measurement of Ki-67.

Colorectal cancer with a high proliferative activity is more likely to respond to chemotherapy (Allegra et al, 2002; Allegra et al 2003; Garrity et al, 2004). Garrity and coworkers (2004) analysed the proliferative activity of colon cancer in the treatment and non treatment group separately. Within treatment group, patients with high ki-67 protein expression showed improved overall survival regardless of whether they received active treatment. According to these results, proliferative markers have the potential to distinguish patients with rapidly proliferating tumors that are likely to respond to chemotherapy from patients with slowly proliferating tumors who may not need aggressive treatment. Selection of those patients, by assessment of the proliferative activity, may be a reliable approach to predict which patients will respond to chemotherapy.

Recently, Valera and co-workers (2005) reported that, using a double immunohistochemical staining procedure and a quantitative technique in a cohort of 106 patients who underwent curative resection for colorectal cancer, an increased Ki-67 proliferation index was associated with poorer survival and it maintained significance as an independent predictor of long-term outcome in a multivariate analysis that included other prognostic factors. This study also showed a significant correlation between proliferative index and several pathological characteristics of colorectal carcinomas, such as tumour differentiation, metastatic disease and local invasiveness in keeping with earlier studies (Porschen et al, 1991; Kubota et al, 1992; Diebold et al, 1994; Kyzer et al, 1997).

1.2.5 Inflammatory disease and other risk factors

Inflammatory bowel disease

Patients with inflammatory bowel disease (IBD) are at increased risk of developing colorectal cancer when compared with the general population (Table 1.2), whose average lifetime risk is 2 to 8 percent (Eaden, 2000; Eaden, 2001). Colorectal cancer represents the major cause of excess morbidity and mortality from malignant disease in patients with ulcerative colitis and to a lesser extent in Crohn's disease. Colorectal cancer was observed in 5.5-13.5 % of all patients with ulcerative colitis and 0.4-0.8 % of patients with Crohn's disease (Pohl, 2000).

Established risk factors include long duration of disease (Eaden, 2001), extensive disease (Ekbom, 1990), young age at onset (Eaden, 2001), the presence of complicating primary sclerosing cholangitis (Brentnall et al, 1996; Kornfeld, 1997), persisting activity of the disease and inadequate pharmacological therapy (Bernstein, 2002).

C-reactive protein concentration

It is of interest therefore, that an increase in the marker of systemic inflammation, such as C-reactive protein is associated with an increase incidence of colorectal cancer in general population (Erlinger et al, 2004).

A recent epidemiologic study has raised the hypothesis that C-reactive protein concentration (CRP) might also be associated with incident colorectal carcinoma and considered as risk factor (Erlinger et al, 2004). This latter finding is supported by clinical observations of an increased risk for colorectal cancer in patients with chronic,

relapsing and remitting inflammatory bowel diseases (Panes, 2001; Munkholm, 2003). Erlinger and co-workers (2004) investigated the possibility that C-reactive protein, as a marker for inflammation, could help identify people at risk for the development of colorectal cancer. C-reactive protein was measured as (baseline) at the beginning of their prospective study. During 11 years follow-up period, a total of 172 subjects develop colorectal cancer, of these 131 had colon cancer and 41 had rectal cancer. For each colorectal cancer case, 1 or 2 case matched controls were found. Those apparently healthy subjects with the higher C-reactive protein concentration (top 25%) had twice the risk of colorectal cancer compared with those with lower C-reactive protein concentration (bottom 25%). However, depending on whether a case-control or cohort study design was used, these results have or have not been confirmed (Gunter et al, 2006; Zhang et al, 2005).

In summary, there is some evidence that the presence of systemic inflammatory response, as evidenced by elevated C-reactive protein concentration, plays a role in the development and or progression of colorectal cancer.

Table 1.1 Established risk factors for colorectal cancer (adapted from DeVita, 2001).

Genetic

Polyposis Syndromes

Familial polyposis coli

Gardner's syndrome

Turcot syndrome (CNS tumours)

Oldfield's syndrome (sebaceous cysts)

Peutz-Jeghers syndrome (hamartomas)

Nonpolyposis syndromes

Lynch syndrome I

Lynch syndrome II (associated extracolonic cancers)

Pre-existing disease and inflammation

Ulerative colitis

Crohn's diseasc

Prior colorectal cancer

Neoplastic polps

Pelivic irradiation

General

Age >40 y

Family history of colorectal cancer

1.2.6 Chemoprevention

Non-steroidal Anti-inflammatory drugs and colorectal cancer

There is evidence that the use of non-steroidal anti-inflammatory drugs (NSAID) is associated with reduced risks of colorectal cancer and adenomatus polyps (Rosenberg, 1991; Peleg, 1994; Giovannucci, 1994). Firstly, several NSAIDs, including aspirin, inhibit the growth of chemically induced colonic tumours in rats and mice (Pollard, 1983; Narisawa, 1983; Reddy, 1990). Secondly, the NSAID sulindac causes the regression of residual rectal polyp in patients with familial adenomatous polyposis and an ileorectal anastomosis (Nugent et al, 1992; Giardiello et al, 1993). Finally, in epidemiological studies, the use of aspirin and NSAIDs was associated with an approximate halving of the risk of developing or dying from colonic or colorectal cancer (Rosenberg, 1991; Thun, 1991).

In 1977, Bennett and co-workers noted increased concentrations of prostaglandins in colorectal cancer tissue when compared with normal colorectal mucosa. Given the pharmacological ability of non-steroidal anti-inflammatory drugs to inhibit the cyclooxygenase (COX-1) enzyme and thereby block prostaglandin synthesis, several studies evaluated the use of non-steroidal anti-inflammatory drugs and demonstrated that they could both prevent and reverse colorectal adenomas and carcinomas in animal models and man (Pollard et al, 1980; Kudo et al, 1980; Kune et al, 1988). Since then several other studies have been published and nearly all have confirmed the original reports. Indeed, the chemopreventive properties of several non-steroidal anti-inflammatory drugs including aspirin (Giovannucci, 1994), indomethacin (Rubio et al, 1989), piroxicam (Pollard et al, 1984), and sulindac (Skinner et al, 1991) have been confirmed.

Clinical experience with non-steroidal anti-inflammatory drugs included a series of case reports (Waddell and Loughry, 1983; Waddell et al, 1989) and randomised trials (Labayle et al, 1991; Giardello et at, 1993), which demonstrated the ability of NSAID including sulindac to reduce the size and number of colorectal polyps occurring in patients with familial adenomatous polyposis (FAP). These findings have important public health implications, as it is likely that the adenoma/carcinoma sequence in familial adenomatous polyposis patients is similar to that of the general population. This concept is supported by recent epidemiological findings of a 40-50% reduction in mortality from colorectal cancer among people regularly taking non-steroidal anti-inflammatory drugs compared with those not taking these agents (Cotton et al, 1996; Smalley et al, 1997; Janne, 2000; Sandler, 2003).

Despite the strong evidence that the use of non-steroidal anti-inflammatory drugs lowers the risk of colorectal cancer, many questions remain. Aspirin was the sole or predominant non-steroidal anti-inflammatory drug evaluated in most of the epidemiological studies to date. However, patterns of use (dose and duration) necessary to prevent colorectal cancer are still unclear. Giovannucci and colleagues (1995) found an increasing protective effect with an increasing duration of regular aspirin use. Although this reduction became significant only after 20 years of use, the relative risk for colorectal cancer began to steadily decline after 5 to 9 years of regular use. Rosenberg and co-workers (1991) found a protective effect when regular use was started in the year before diagnosis and found a weak effect of duration of use when any regular use was compared with regular use over five or more years. Although these

independent lines of research all support the link between NSAID use and a reduction in colorectal cancer, the pharmacological basis of NSAID protection remains unclear.

It is likely that these mechanisms are related, at least in part, to those underlying the anti-inflammatory properties of non-steroidal anti-inflammatory drugs, that is, their ability to inhibit the cyclooxygenase enzymes (Smalley et al, 1997), which reduces the levels of tissue-specific prostaglandins (PG) (Figure 1.2), the mediators of the inflammatory response (Funk, 2001). Cyclo-oxygenase (COX) is an enzyme that is responsible for the conversion of arachidonic acid into prostaglandins (PG).

An inducible form of cyclo-oxygenase was recognized in early 1990s (Xie et al, 1991; Masferrer et al, 1992). This was confirmed and led to the classification of two isoenzymes (Table 1.1), COX-1, the constitutive form, is expressed in many tissues to regulate and maintain normal cellular function. In contrast, COX-2 is expressed only after stimulation via a variety of different growth factors, cytokines and mitogens (Smith et al, 2001). Aspirin and other NSAIDs inhibit the activity of both isoforms; the therapeutic anti-inflammatory effects are attributed to COX-2 inhibition, whereas the side-effects of these drugs (gastrointestinal inflammation, ulceration, prolonged bleeding time) seem to be due primarily to the inhibition of COX-1 (FitzGerald et al, 2001; Chan et al, 2002).

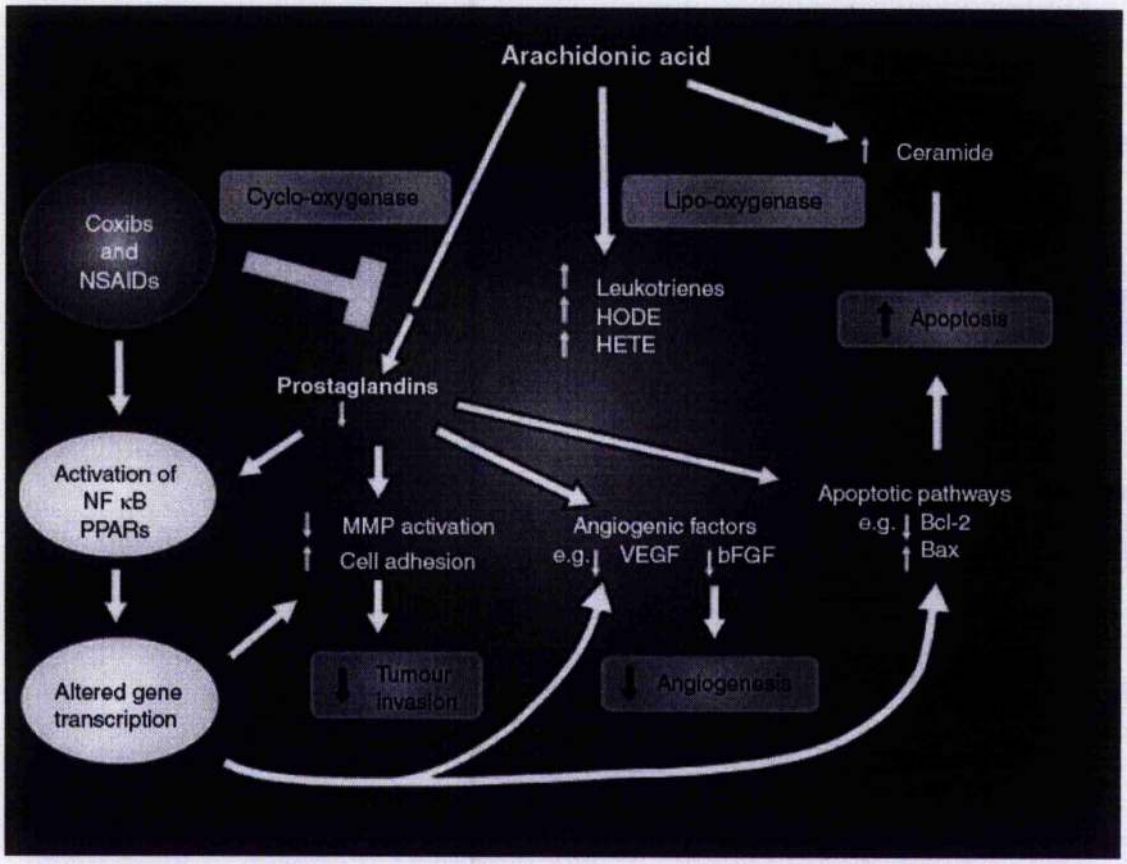


Figure 1.2 Potential mechanistic pathways that may account for some of the anticancer effects of the non-steroidal anti-inflammatory drugs (NSAIDs) and cyclo-oxygenase 2 inhibitors (coxibs) (Adapted from Church, 2003).

Protection is probably multifactorial and could be related to the ability of NSAIDs to arrest colorectal carcinogenesis at several stages. The specific mechanisms of action of COX-2 inhibitors as anticancer agents have yet to be established. The inhibition of cyclo-oxygenase and reduction in tissue prostaglandin levels in human subjects have been established (FitzGerald, 2001), but the downstream cellular events are not as clear. An impact of the coxibs on angiogenesis (Jones, 1999) and apoptosis (Richter, 2001) has been observed. In addition, there is some evidence to suggest that Cox-2 inhibitors have effects on cellular proliferation and adhesion, breakdown of the extracellular

matrix, and modulation of immune surveillance (Dannenberg, 1999; Pan, 2001;Yamazaki, 2002).

COX-2 inhibitors may prove to be attractive chemopreventive agents because selective inhibition may prevent cancer while avoiding the complication of bleeding and gastric irritation (FitzGerald, 2001; Chan, 2002). However, to date their efficiency in humans is not well defined.

Table 1.2 Cyclo-Oxygenase enzymes (Adapted from Church, 2003).

Cyclo-oxygenase-1		Cyclo-oxygenase-2
Expression	Constitutive	Constitutively expressed only in brain, lung, kidney
	Found in all tissues	Rapidly induced in inflammatory tissue (1-3 h) induced by cytokines and growth factors (IL-2,TNF-α) inhibited by anti-inflammatory cytokins (IL-10)
Functions	Housekeeping function in platelet, stomach, kidneys, and endothelium	Inflammatory process in macrophages, leucocytes, fibroblasts, and endothelium
Inhibition	Aspirin and non-steroidal anti-inflammatory drugs	Aspirin, non-steroidal anti-inflammatory drugs and glucocorticoids

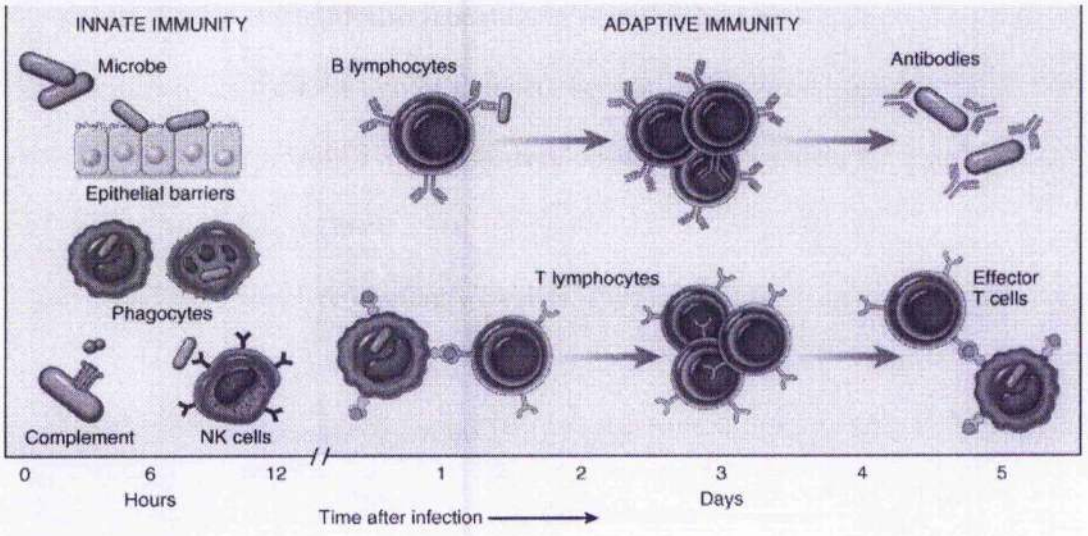
COX = Cyclo-oxygenase, IL-1= Interleukin 1, IL-10 = Interleukin 10, TNFα = Tumour necrosis factor alpha.

1.3 Host Immune Response and Colorectal Cancer

1.3.1 Basis of immunology

The physiologic function of the immune system is to protect individuals from infectious pathogens. The mechanisms that are responsible for this protection fall into two broad categories (Figure 1.3). Innate immunity (native immunity) refers to defence mechanisms that are present even before infection and have evolved to specifically recognize microbes and protect multicellular organisms against infections. Adaptive immunity (specific, immunity) consists of mechanisms that are stimulated by (adapt to) microbes and are capable of also recognizing nonmicrobial substances, called antigens. Innate immunity is the first line of defence, because it is always ready to prevent and eradicate infections. Adaptive immunity develops later after exposure to microbes and is even more powerful in combating infections. By convention, the term "immune response" refers to adaptive immunity (Abbas, 2005).

Figure 1.3 Innate and adaptive immunity (Abbas, 2005).



Innate immunity

The major components of innate immunity are epithelial barriers that block entry of environmental microbes, phagocytic cells (mainly neutrophils and macrophages), natural killer (NK) cells, and several plasma proteins, including the proteins of the complement system. Phagocytes are recruited to sites of infection, resulting in inflammation, and here the cells ingest the microbes and are then activated to destroy the ingested pathogens. Phagocytes recognize microbes by several membrane receptors (Abbas, 2005).

Complement proteins are some of the most important plasma proteins of the innate immune system. Recall that in innate immunity, the complement system is activated by binding to microbes using the alternative pathways; in adaptive immunity, it is activated by binding to antibodies using the classical pathway. Other circulating proteins of innate immunity are mannose-binding lectin and C-reactive protein, both of which coat microbes for phagocytosis and complement activation (see section 1.3.6). Lung surfactant is also a component of innate immunity, providing protection against inhaled microbes.

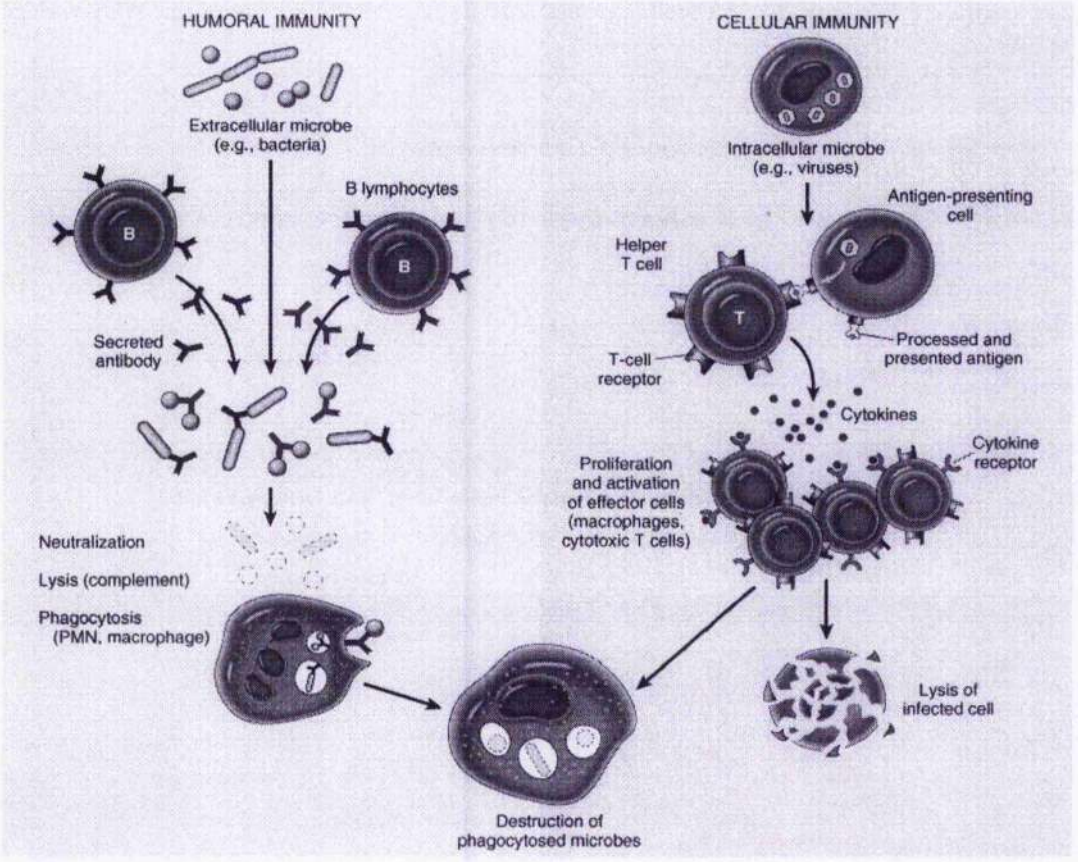
Adaptive immunity

The adaptive immune system consists of lymphocytes and their products, including antibodies. The receptors of lymphocytes are much more diverse than those of the innate immune system, but lymphocytes are not inherently specific for microbes, and they are capable of recognizing a vast array of foreign substances.

1.3.2 Humoral and Cell-mediated immunity

There are two main types of adaptive immunity—cell-mediated (or cellular) immunity, which is responsible for defence against intracellular microbes, and humoral immunity, which protects against extracellular microbes and their toxins (Fig. 1.4). Cellular immunity is mediated by T (thymus-derived) lymphocytes, and humoral immunity is mediated by B (bone marrow-derived) lymphocytes and their secreted products, antibodies. Both these mechanisms of adaptive immunity are capable of causing injury to the host and subsequent disease.

Figure 1.4 Humoral and cell-mediated immunity (Abbas, 2005).



T Lymphocytes

T lymphocytes are generated from immature precursors in the thymus. Mature, naive T lymphocytes are found in the blood, where they constitute 60% to 70% of lymphocytes, and in T- lymphocytes zones of peripheral lymphoid organs, such as the paracortical areas of lymph nodes and periarteriolar sheaths of the spleen. The segregation of naive T lymphocytes to these anatomic sites is because the cells express receptors for chemoattractant cytokines (chemokines) that are produced only in these regions of lymphoid organs (Cyster, 1999; Mebius, 2003) Each T lymphocytes is genetically programmed to recognize a specific cell-bound antigen by means of an antigen-specific T-cell receptor (TCR) (Davis, 1998). In approximately 95% of T lymphocytes, the TCR consists of a disulfide-linked heterodimer made up of α and β polypeptide chain (Figure 1.5), each having a variable (antigen-binding) and a constant region. The α β TCR recognizes peptide antigens that are displayed by major histocompatibility soluble antigens; therefore, presentation of processed, membrane-bound antigens by antigen-presenting cells is required for induction of cell-mediated immunity. T- lymphocytes receptors are capable of recognizing a very large number of peptides; each T lymphocytes expresses TCR molecules of one structure and specificity.

A minority of mature T lymphocytes express another type of TCR composed of γ and δ polypeptide chains (Hayday, 1999). The γ δ TCR recognizes peptides, lipids, and small molecules, without a requirement for display by MHC proteins. These T cells tend to aggregate at epithelial surfaces, such as the mucosa of the respiratory and gastrointestinal tracts, suggesting that these cells are sentinels that protect against microbes that try to enter through these epithelia. Another small subset of T lymphocytes expresses markers that are found on natural killer (NK) cells; these cells

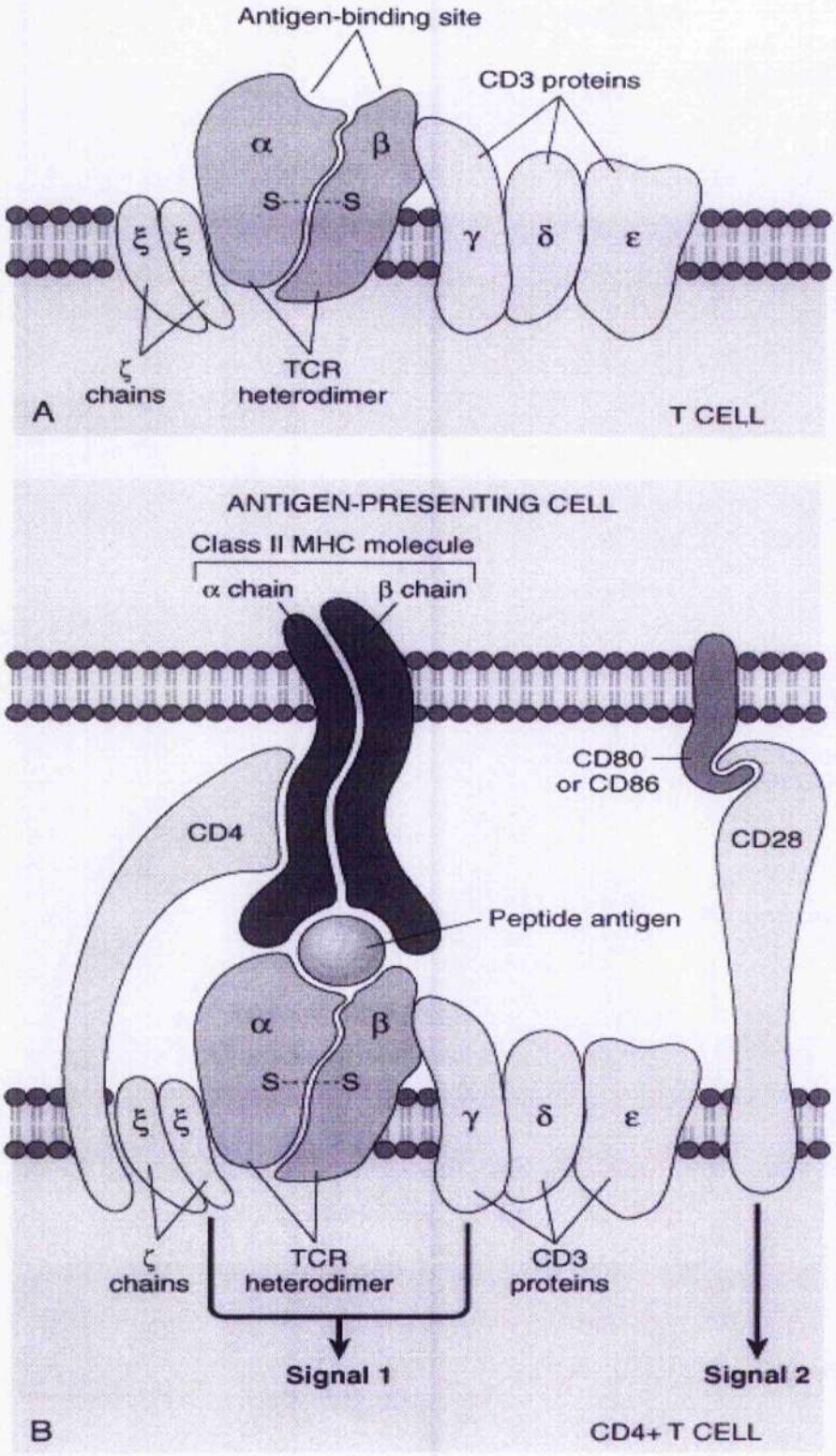
are called NK-T lymphocytes. NK-T lymphocytes express a very limited diversity of TCRs, and they recognize glycolipids that are displayed by the MHC-like molecule CD1. The functions of NK-T lymphocytes are also not well defined.

T lymphocytes also express a number of non-polymorphic, function-associated molecules, also called accessory molecules, including CD4, CD8, CD2, integrins, and CD28. CD4 and CD8 are expressed on two mutually exclusive subsets of α β T lymphocytes. CD4 is expressed on approximately 60% of mature CD3+ T lymphocytes, whereas CD8 is expressed on about 30% of T lymphocytes. These T- lymphocytes membrane-associated glycoproteins serve as co-receptors in T- lymphocytes activation. During antigen presentation, CD4 molecules bind to the non-polymorphic portions of class II MHC molecules expressed on antigen-presenting cells (Figure 1.5).

In contrast, CD8 molecules bind to class I MHC molecules. CD4 and CD8 are required to initiate signals that activate T lymphocytes that recognize antigens. Because of this requirement for co-receptors, CD4+ helper T lymphocytes can recognize and respond to antigen only in the context of class II MHC molecules, whereas CD8+ cytotoxic T lymphocytes recognize cell-bound antigens only in association with class I MHC molecules. It is now well established that T lymphocytes need two signals for activation. Signal 1 is provided when the TCR is engaged by the appropriate MHC-bound antigen, and the co-receptors CD4 and CD8 bind to MHC molecules. Signal 2 is delivered by the interaction of the CD28 molecule on T lymphocytes with the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) expressed on antigen-presenting cells (Figure 1.5). The importance of co-stimulation by this pathway is attested to by the fact that, in the absence of signal 2, the T cells fail to respond, undergo apoptosis, or

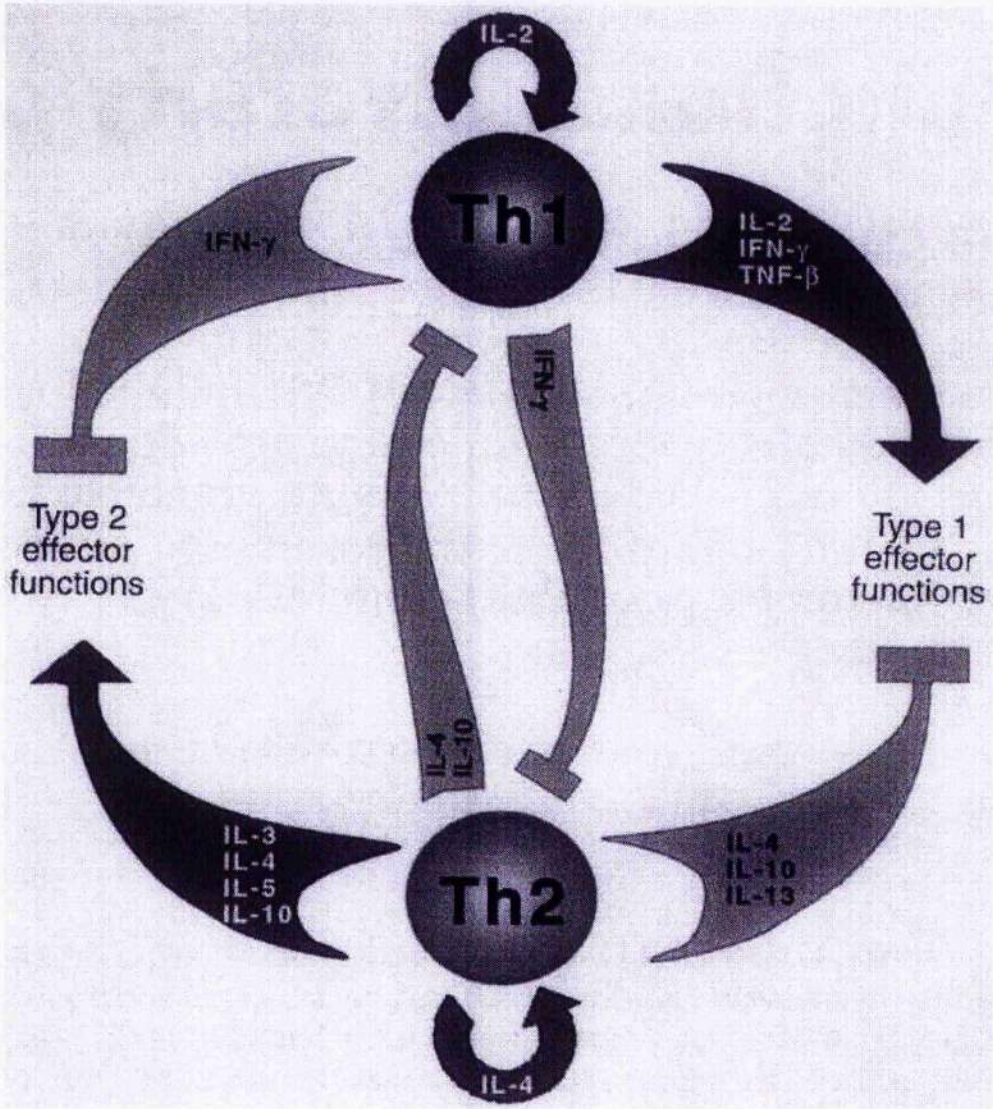
become unreactive (Lenschow et al, 1996). When T lymphocytes are activated by antigen and co-stimulators, they secrete locally acting proteins called cytokines (described below). Under the influence of a cytokine called interleukin-2 (IL-2), the T lymphocytes proliferate, thus generating a large number of antigen-specific lymphocytes. Some of these cells differentiate into effector cells, which perform the function of eliminating the antigen that started the response. Other activated cells differentiate into memory cells, which are long-lived and poised to respond rapidly to repeat encounters with the antigen.

Figure 1.5 **T-cell receptor (TCR) complex (Abbas, 2005).**



CD4+ and CD8+ T lymphocytes perform distinct but somewhat overlapping effectors functions (von Andrian et al, 2000). The CD4+ T lymphocytes can be viewed as a master regulator by secreting cytokines; CD4+ T lymphocytes influence the function of virtually all other cells of the immune system, including other T lymphocytes, B lymphocytes, macrophages, and NK cells. The central role of CD4+ T lymphocytes is tragically illustrated when the human immunodeficiency virus cripples the immune system by selective destruction of this T- lymphocytes subset. In recent years, two functionally distinct populations of CD4+ helper lymphocytes have been recognized on the basis of the different cytokines they produce (Figure 1.6) (Abbas, 1996). The T-helper-1 (T_H 1) subset synthesizes and secretes IL-2 and interferon-γ (IFN-γ) but not IL-4 or IL-5, whereas T_H 2 cells produce IL-4, IL-5 and IL-13 but not IL-2 or IFN-γ. This distinction is significant because the cytokines secreted by these subsets have different effects on other immune cells. The T_H 1 subset is involved in facilitating delayed hypersensitivity, macrophage activation, and synthesis of opsonizing and complement-fixing antibodies. The T_H 2 subset aids in the synthesis of other classes of antibodies, notably IgE (mediated by IL-4 and IL-13) and in the activation of eosinophils (mediated by IL-5). CD8+ T lymphocytes function mainly as cytotoxic lymphocytes to kill other cells but, similar to CD4+ T lymphocytes, they can secrete cytokines, primarily of the T_H 1 type.

Figure 1.6 The cytokines produced by Th1 and Th2 lymphocyte populations (Abbas, 1996).



B Lymphocytes

B lymphocytes develop from immature precursors in the bone marrow. Mature B lymphocytes constitute 10% to 20% of the circulating peripheral lymphocyte population and are also present in peripheral lymphoid tissues such as lymph nodes, spleen, or tonsils and extralymphatic organs such as the gastrointestinal tract. In lymph nodes, they are found in the superficial cortex. In the spleen, they are found in the white pulp. At both sites, they are aggregated in the form of lymphoid follicles, which on activation

develop pale-staining germinal centers. B lymphocytes are located in follicles, the B-lymphocytes zones of lymphoid organs, because the cells express receptors for a chemokine that is produced in follicles (Cyster, 1999; Mebius, 2003).

B lymphocytes recognize antigen via the B- lymphocytes antigen receptor complex. Immunoglobulin M (IgM) and (IgD) D, present on the surface of all naive B cells, constitute the antigen-binding component of the B-cell receptor complex. After antigenic stimulation, B lymphocytes form plasma cells that secrete immunoglobulins, which are the mediators of humoral immunity.

B lymphocytes may be activated by protein and non-protein antigens. The end result of B- lymphocytes activation is their differentiation into antibody-secreting cells, called plasma cells. Antibody-secreting cells reside in lymphoid organs and mucosal tissues, and some plasma cells may migrate to the bone marrow and live for many years in this tissue. Secreted antibodies enter mucosal secretions and the blood and are able to find, neutralize, and eliminate antigens. B- lymphocytes responses to antigens require help from CD4+ T lymphocytes (Clark et al, 1994). Helper T lymphocytes activate B lymphocytes by engaging CD40, a member of the tumour necrosis factor (TNF)-receptor family, and by secreting cytokines. Activated helper T lymphocytes express CD40 ligand, which specifically binds to CD40 expressed on B lymphocytes (Clark et al, 1996). This interaction is essential for B- lymphocytes maturation and secretion of IgG, IgA, and IgE antibodies.

Natural Killer Cells

NK cells make up approximately 10% to 15% of the peripheral blood lymphocytes and do not bear T- lymphocytes receptors or cell surface immunoglobulins. Morphologically, NK cells are somewhat larger than small lymphocytes, and they contain abundant azurophilic granules. Hence, they are also called large granular lymphocytes. NK cells are endowed with an innate ability to kill a variety of tumour cells, virally infected cells, and some normal cells, without previous sensitization (Cerwenka et al, 2001). These cells are part of the innate immune system, and they may be the first line of defence against viral infections and, perhaps, some tumours. NK cells do not rearrange T- lymphocytes receptor genes and are CD3 negative. Two cell surface molecules, CD16 and CD56, are widely used to identify NK cells. CD16 is the Fc receptor for IgG and it endows NK cells with another function, the ability to lyse IgG-coated target cells. This phenomenon, known as antibody-dependent cell-mediated cytotoxicity.

The functional activity of NK cells is regulated by a balance between signals from activating and inhibitory receptors. The activating receptors stimulate NK cell killing by recognizing ill-defined molecules on target cells, some of which may be viral products; the inhibitory receptors inhibit the activation of NK cells by recognition of self-class I MHC molecules. The class I MHC-recognizing inhibitory receptors on NK cells are aptly called killer inhibitory receptors. They are biochemically distinct from T-cell receptors. It is believed that NK cells are inhibited from killing normal cells because all nucleated normal cells express self-class I MHC molecules (Ochsenbein, 2002).

NK cells also secrete cytokines, such as IFN-γ, TNF, and granulocyte macrophage colony-stimulating factor (GM-CSF). IFN-γ activates macrophages to destroy ingested microbes, and thus NK cells provide early defence against intracellular microbial infections. IFN-γ also promotes the differentiation of naive CD4+ T- lymphocytes into T_H 1 lymphocyte. Thus, activation of NK cells early in the immune response can favour induction of delayed hypersensitivity and secretion of opsonising antibodies by promoting the development of T_H 1 lymphocytes. The activity of NK cells is regulated by many cytokines, including IL-2, IL-15, and IL-12. IL-2 and IL-15 stimulate proliferation of NK cells, whereas IL-12 activates killing and secretion of IFN- γ (Ochsenbein, 2002).

1.3.3 Cytokines: messenger molecules of the immune system

Cytokines are intercellular signalling polypeptides produced by activated cells mediating cell-cell communication during inflammatory and immune responses. Cytokines term includes the previously designated lymphokines (lymphocyte-derived), monokines (monocyte-derived), and several other polypeptides that regulate immunologic, inflammatory, and reparative host responses. Molecularly defined cytokines are called interleukins, implying that they mediate communications between leukocytes. Most cytokines have a wide spectrum of effects, and some are produced by several different cell types.

The cytokines that are produced during and participate in inflammatory processes are the chief stimulators of the production of acute-phase proteins. These inflammation-associated cytokines include interleukin-1, interleukin-6, tumour necrosis factor (alpha),

interferon (gamma), transforming growth factor (beta), (Kushner, 1993) and possibly interleukin-8 (Wigmore et al, 1997).

Interleukin-1 has a multifunctional role. It stimulate the production of lymphocyte-activating factor, endogenous pyrogen, leucocyte endogenous mediator, osteoclast activating factor, B-cell stimulating factor, catabolism and proteolysis-inducing factor (Dinarello et al, 1988).

Interleukin-2 promotes proliferation and differentiation of helper T lymphocytes, cytotoxic T lymphocytes, and B-lymphocytes. Interleukin-2 is in turn produced by CD4+ and CD8+ lymphocytes and acts as an immune stimulant. It has also been reported to activate lymphocytes and kill tumour cells in vitro and in vivo (Smith, 1988). Consequently, interleukin-2 participates in both cell-mediated immunity and humoral immunity, and is probably necessary for both primary and secondary adaptive immune responses. Interleukin-2 may also augment innate or "natural" immunity by stimulating natural killer (NK) cells and monocytes. Interleukin-2 can also induce the classic signs of delayed-type hypersensitivity, even in the absence of specific antigen. (Caligiuri et al, 1993).

Tumour necrosis factor functions (TNF) as a pyrogen, activates the clotting system and stimulates production of the acute phase proteins by the liver. Tumour necrosis factor induces expression of the adhesion molecules, which promote adhesion, and migration of leucocytes from the blood vessels to the extra-vascular compartment. It also activates white blood cells leading to the production by macrophages of various cytokines, including interleukin-6 and tumour necrosis itself (Woolf, 1998).

Interleukin-6 is the chief stimulator of the production of most acute-phase proteins (Gauldie et al, 1987), whereas the other implicated cytokines influence subgroups of acute-phase proteins. It is released by a number of cells, including monocytes, fibroblasts and endothelial cells.

Interleukin-6 is commonly produced at local tissue sites and released into circulation in almost all situations of homeostatic disruption typically including endotoxemia, trauma, and acute infections (Kishimoto et al, 1992; Zitnik et al, 1993). In addition to its critical participation in the generation of immunity against chronic intracellular infections, circulating IL-6, together with other alarm cytokines TNF-α and IL-1, is known to be required for the induction of acute phase reactions composed of fever, corticosterone release, and hepatic production of acute phase proteins many of which are protease inhibitors (Baumann et al, 1994; Xing et al, 1995). Overall, the induction by IL-6 of these acute phase reactions has been regarded as part of an attempt to maintain homeostasis. However, it still remains unclarified whether during local or systemic acute inflammatory responses, IL-6 is also directly involved in the modulation of other aspects of inflammation, particularly cytokine responses and tissue inflammatory infiltration.

During local or systemic acute inflammatory responses, IL-6 is involved in the induction of acute phase reactions and controlling the level of acute inflammatory responses by downregulating the expression of proinflammatory cytokines and upregulating antiinflammatory molecules including IL-1 receptor antagonist protein (IRAP), TNF soluble receptor (TNFsR), and extrahepatic protease inhibitors. During

chronic diseases, particularly intracellular viral or bacterial infections, IL-6 is involved not only in the elicitation of acute phase reactions but the development of specific cellular and humoral immune responses including end-stage B cell differentiation, immunoglobulin secretion and T cell activation.

General Properties of Cytokines

Although cytokines have many diverse actions, all of them share some important properties. The actions of cytokines are pleiotropic, meaning that any one cytokine may act on many cell types and mediate many effects. For example, IL-2, initially discovered as a T-cell growth factor, is known to affect the growth and differentiation of B cells and NK cells as well. Cytokines are also often redundant, meaning that different cytokines may stimulate the same or overlapping biologic responses.

Cytokines induce their effects in three ways: (1) They act on the same cell that produces them (autocrine effect), such as occurs when IL-2 produced by antigen-stimulated T cells stimulates the growth of the same cells; (2) they affect other cells in their vicinity (paracrine effect), as occurs when IL-7 produced by bone marrow or thymic stromal cells promotes the maturation of B-cell progenitors in the marrow or T-cell precursors in the thymus, respectively; and (3) they affect many cells systemically (endocrine effect), the best examples in this category being IL-1 and TNF, which produce the systemic acute-phase response during inflammation (Gabay and Kushner, 1999).

Cytokines mediate their effects by binding to specific high-affinity receptors on their target cells. For example, IL-2 activates T cells by binding to high-affinity IL-2 receptors (IL-2R). Blockade of the IL-2R by specific anti-receptor monoclonal

antibodies prevents T-cell activation. This observation is the basis for the use of anti-IL-2R antibodies to control undesirable T-cell activation, as in transplant rejection. The knowledge gained about cytokines has practical therapeutic ramifications. First, by inhibiting cytokine production or action, it may be possible to control the harmful effects of inflammation or tissue-damaging immune reactions. Patients with rheumatoid arthritis often show dramatic responses to TNF antagonists, an elegant example of such therapy. Second, recombinant cytokines can be administered to enhance immunity against cancer or microbial infections (immunotherapy), (Abbas, 2005).

1.3.4 Tumour Immunity

The biological malignancy of human cancer is determined by the net effects of biological malignancy of tumor cells and host factors. The immune and inflammatory reactions are representatives of the host factors. In human cancers, however, such immune reactions are generally insufficient because of weak immunogenicity of cancer cells or impaired immunity in cancer-bearing patients (Villunger et al, 1999). The presumptive mechanisms of these included absence of tumor-specific antigens, down-regulation of MHC class I molecule expression (Natali et al, 1989), or secretion of immunosuppressive cytokines (Barth et al, 1996). Recently, functional inactivation of potentially tumor-reactive T cells was analyzed as an important mechanism of immune evasion (Lee et al, 1999).

Tumour antigens

For the adaptive immunity against tumours, antigens are needed to trigger a specific immune response. Ideal antigens should be able to elicit specific B or T cell responses or both, with T cell responses including recognition of HLA class II-restricted epitopes.

Tumour associated antigens (TAA) that can be recognised on tumour cells by T lymphocytes form a heterogenous group. They are generally divided into three categories (Rosenberg et al, 1996; Boon et al, 1996). The first consists of unique antigens that are specific for each single tumour, usually derived from mutated proteins, and can be recognised either by HLA class I or HLA Class II restricted T lymphocytes, according to their length and aminoacid sequence (Rosenberg et al, 1996; Boon et al, 1996; Pardoll and Topalian, 1998). TAA shared with other tumours but predominantly expressed by malignancies form a second category; these are usually transcriptionally reactivated genes that are not normally expressed (e.g. genes from the MAGE family in various tumours and testicular tissue), previously known as embryonic antigens and recently referred to as cancer testis (CT) antigens. A third category is that of differentiation antigens derived from the tissue of origin of the tumour (e.g. gp100, Melan-A, and tyrosinase in melanomas). Surprisingly enough, these normal antigens turned out to include some of the TAA most frequently recognised by the immune system (Rosenberg et al, 1996). Yet other antigens, have been considered for immunotherapy of cancer. Examples of these are overexpressed antigens that are also present in normal tissues (e.g. CEA) (Bremers et al, 1995). Moreover, oncogene and tumour suppressor gene products that play an essential role in the development of malignancies, can generate epitopes able to induce immune responses (e.g. mutated RAS, p53), (Rosenberg et al, 1996; Boon et al, 1996; Pardoll and Topalian, 1998). Since some tumour histotypes, like pancreatic and lung cancers, bear such mutated genes with a high frequency, such antigens may represent interesting targets. Finally, viral products in virus associated tumours can deliver a potent and effective stimulus to the immune system (hepatitis B virus), (Chang et al, 1997), Human papilloma virus (Ressing et al, 1996).

It is of note that only recently HLA class II-restricted epitopes have been clearly defined and that most of them belong to the unique group of antigens that derive by point mutation from already known proteins (Toes et al, 1999).

T- Lymphocytes

Most efforts put into the development of tumour immunology have focused on cytotoxic T lymphocytes (CTL) mediated immunity, since these cells are regarded as the most important effectors cells against tumours. Recognition of tumour antigens by cytotoxic T lymphocytes (CTL) is the result of a complex process (Figure 1.7).

In this system an antigen is processed inside the target or antigen presenting cell (APC) into small peptide fragments that are then presented at the cell surface within the groove of the Major Histocompatibility Complex (MHC, HLA in humans) Class I molecules. This complex is then recognised by a T cell receptor (TCR) present on cytotoxic T lymphocyte (CTL). This T cell receptor (TCR) must be fitting to both the HLA allele present on the cell, and to the peptide presented (Davis et al, 1998). Though underestimated during the last few years, MHC Class II restricted recognition is also very important for the induction of antitumour cytotoxic T lymphocyte (CTL) which are activated through a (CD4 mediated) T helper response (Pardoll and Topalian, 1998).

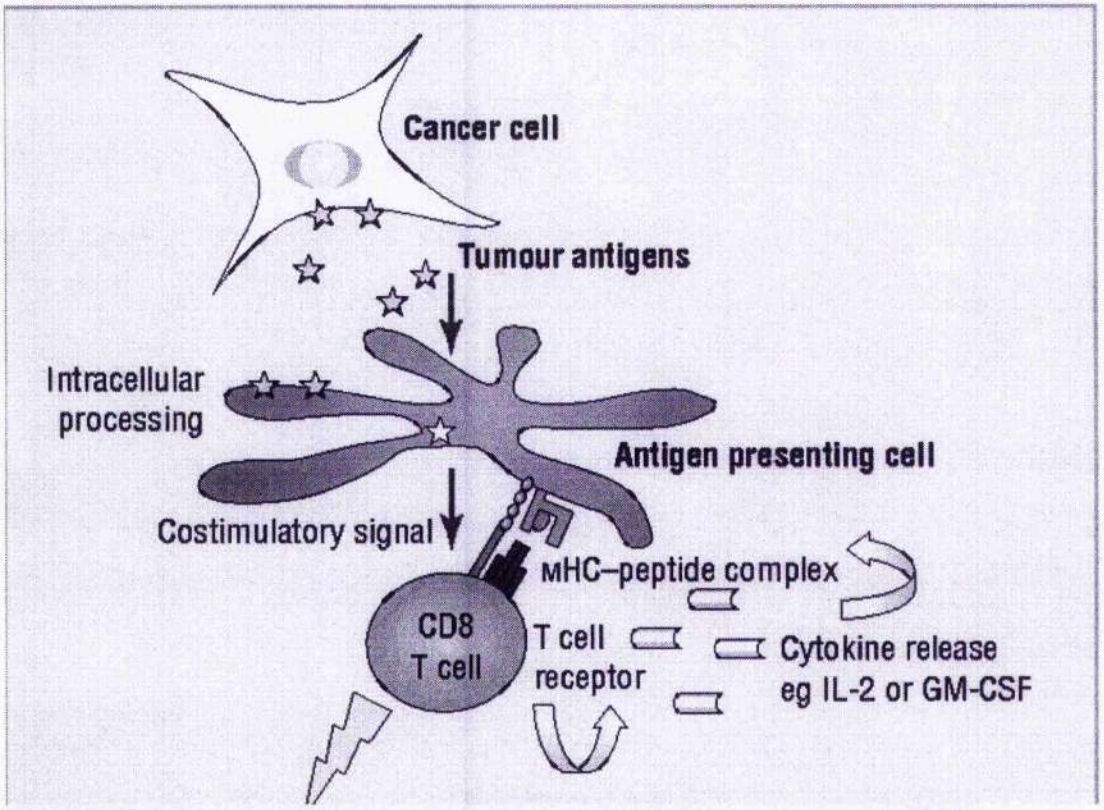


Figure 1.7 Anti tumour immune response. (Adapted by Armstrong, 2001).

The outcome of antigen recognition though, is not necessarily tumour cell killing. Inflammation or destruction, occurring during infection, come with cytokine-mediated signals (e.g. TNF-α and GM-CSF), resulting in activation of T-lymphocytes. Lack of these signals may result in tolerance, as is usually the case for the majority of antigens physiologically present in the body.

1.3.5 Tumour T-lymphocytes infiltration

Several studies clearly support the protective role of a pronounced tumour lymphocytic infiltrate in colorectal cancer (Jass,1986; Harrison et al, 1994; Ropponen et al, 1997; Naito et al, 1998; Nielsen et al, 1999; Guidoboni et al, 2001). The results from the above studies on this issue are difficult to compare because the histological criteria used to define intra-tumour lymphocytes infiltrates differ from study to study, both qualitatively and quantitatively (Compton et al, 2000). Variations in the pathological definition of lymphocytic infiltrate presumably explain variations in study results, because lymphocytic infiltrate are biologically heterogeneous and can originate through different mechanisms, reflecting diversities in tumour biology and tumour-host interaction. To avoid confusion, pathological evaluation of intra-tumour lymphocytic infiltrate should take into careful consideration some important parameters, such as cellular T-lymphocytes composition and anatomical localisation.

From an immunologist's point of view, the type of immune effectors that can more reliably be considered as a sign of a systemic anti-tumour immune response are cytotoxic T lymphocytes (CTLs), classically CD8 T-lymphocytes. T- lymphocytes are the main effector arm of acquired and antigen-specific cellular immune responses, and play a central role both in the control of viral infections and in immunity against cancer (Melief et al, 1991; Riddell et al, 1995). Indeed, also in colorectal cancer several studies have focused on lymphocytes and have indicated that the presence of lymphocytic infiltrates might have a positive prognostic role (Naito et al, 1998; Nielsen et al, 1999; Guidoboni et al, 2001). These results are difficult to evaluate and to compare, because in many cases, especially in early studies, no functional characterization of infiltrating lymphocytes was performed or specific staining for CD4+ and CD8+ T-lymphocytes.

Distinction among different subsets of lymphocytes is probably important, because the lamina propria of normal colorectal mucosa is rich in B cells (Lee et al, 1988). Moreover, different studies show substantial differences in other important parameters with potential prognostic value, such as the anatomical distribution of lymphocytes within the tumour tissue.

When evaluating the presence of lymphocytic infiltrates, authors use different criteria to include or exclude them in their analysis, depending on the anatomical localization of the immune effectors within the tumour tissue. Different authors have focused on different types of lymphocytic infiltrates, including: (a) the presence of a peri-tumoural lymphocytic stromal reaction, forming a continuous mantle or cuff at the invasive margin of the tumour (Jass, 1986), (b) the presence of so-called Crohn's like lymphoid reactions, defined as lymphoid aggregates, often with germinal centres, surrounding the periphery of invasive carcinoma (Graham , 1990; Harrison et al, 1994), (c) the presence of lymphocytic infiltrates within the tumour stroma (Ropponen et al, 1997; Nielsen et al, 1999), (d) the presence of lymphocytic infiltrates directly infiltrating within the tumour epithelium (Naito et al, 1998; Guidoboni et al, 2001).

Prognostic role of intra-epithelial T-lymphocytes infiltrates

A key to understand the prognostic and complex role of lymphocytic infiltrates in colorectal cancer could reside in accurate and quantitative classification of inflammatory infiltrates, and their anatomical localization within the tumour tissues (intra-epithelial vs. stromal or peri-tumoural). Indeed, this approach has recently been undertaken by Naito and co-workers (1998) who have evaluated the prognostic role of CD8+ T-lymphocytes infiltrates in primary colorectal carcinomas, dividing CD8+ T-

lymphocytes in three groups: (a) peri-tumoural, when distributed along the invasive margin of the tumour; (b) stromal, when infiltrating the tumour stroma; and (c) intra-epithelial, when infiltrating within cancer cell nests and taking direct contact with tumour cells. In this study on 131 patients, the presence of peri-tumoural and stromal CD8 T-lymphocytes was not associated with improved prognosis in multivariate analysis, while the presence of intra-epithelial CD8+ T-lymphocytes was a strong predictor of better survival independently of Duke's stage. These data have now been confirmed by an independent studies on 109 colon cancer patients (Guidoboni et al, 2001), and show two important features: (a) a clear and objective method to classify lymphoid infiltrates, where intra-epithelial CD8+ T-lymphocytes are readily identified within epithelial cell nests; (b) compatibility with current theories on anti-tumour immunity, since they focus on a specific category of immune effectors that are known to play a central role in anti-tumour immune responses (CD8+ T-lymphocytes) and evaluate their actual distribution among neoplastic cells.

A better understanding of the role and nature of intra-epithelial CD8+ T-lymphocytes could benefit from their detailed functional characterisation. Functional characterization of Tumour infiltrating lymphocytes has proved to be an important feature in renal cell carcinoma, where the prognostic role of intra-tumour CD8 T-lymphocytes is dependent on a high proliferative activity, as defined by immunohistochemistry for the proliferation antigen Ki-67 (Nakano et al, 2001). It has been shown that intra-epithelial T-lymphocytes in colorectal carcinoma display a higher Ki-67 labelling index (Naito et al, 1998), and higher expression of molecules involved in target cell killing, such as perforin and granzyme B (Guidoboni et al, 2001; Dolcetti et al, 1999), when compared

with those localised in the tumour stroma or the adjacent normal mucosa, suggesting that they are actually active effectors.

1.3.6 Systemic Inflammatory Response

A local injury (e.g. infection, necrosis, tumour growth) of sufficient magnitude will lead to a series of systemic changes in body haemostasis, involving many organ systems, distant from the site of inflammation (Heinrich et al, 1990). Notable among these systemic changes is the acute-phase protein response, so-called since the discovery that C-reactive protein was increased in the plasma of patients during the acute phase of pneumococcal pneumonia (Tillett, 1930). The term, acute phase response, has continued to be used despite the realisation that this response accompanies both acute and chronic inflammatory disorders. More correctly this response should be termed the systemic inflammatory response (Gabay and Kushner, 1999).

The acute-phase proteins are a family of proteins synthesized solely by the liver. An acute-phase protein has been defined as one whose plasma concentration increases (positive acute-phase proteins) or decreases (negative acute-phase proteins) by at least 25 percent during an inflammatory disorder (Morley, 1982). This response is associated with characteristic metabolic changes in several other systems that include hematological, endocrinological, and immunological dysfunction. These changes are believed to be mediated by proinflammatory cytokines, notably the hepatocyte-stimulating cytokine, interleukin-6 (Heinrich, 1990). The changes in the concentrations of acute-phase proteins are due largely to changes in their production by hepatocytes. The magnitude of the increases varies from about 50 percent in the case of

ceruloplasmin and several complement components to as much as 1000-fold in the case of C-reactive protein and serum amyloid A (Figure 1.8).

Table 1.3 Human Acute-Phase Proteins.

Proteins whose plasma concentrations increase
Complement system
C3
C4
C9
Factor B
C1 inhibitor
Coagulation and fibrinolytic system
Fibrinogen
Plasminogen
Tissue plasminogen activator
Urokinase
Protein S
Plasminogen-activator inhibitor 1
Antiproteases
α-1 Protease inhibitor
α-1 Antichymotrypsin
Inter-α-trypsin inhibitors
Transport proteins
Ceruloplasmin
Haptoglobin
Hemopexin
Participants in inflammatory responses
Secreted phospholipase A2
Interleukin-1-receptor antagonist
Granulocyte colony-stimulating factor
Others
C-reactive protein
Serum amyloid A
α-Acid glycoprotein
Ferritin
Proteins whose plasma concentrations decrease
Albumin
Transferrin
α-fetoprotein
Thyroxin-binding globulin
Insulin-like growth factor I
Factor XII

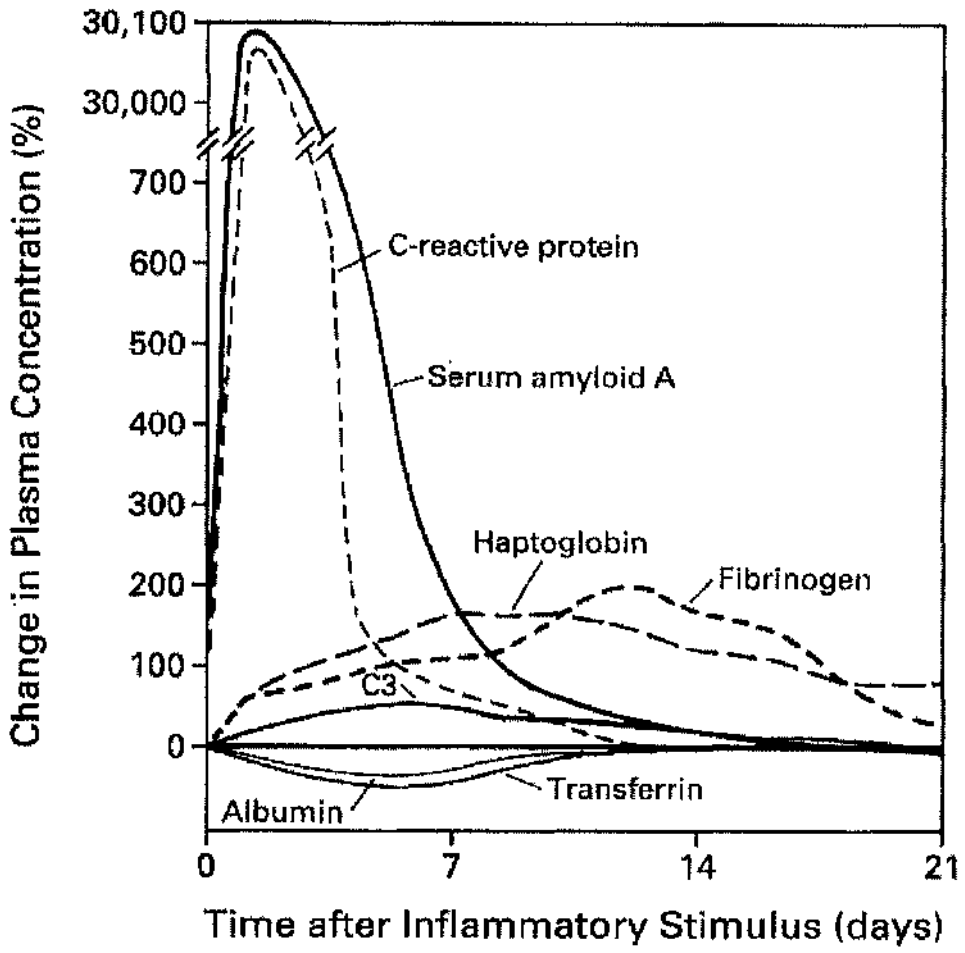


Figure 1.8 Characteristic Patterns of Change in Plasma Concentrations of Some Acute-Phase Proteins after a Moderate Inflammatory Stimulus (Adapted from Gabay and Kushner, 1999).

Although the concentrations of multiple components of acute-phase response commonly increase together, not all of them increase uniformly in all patients with the same illness. Thus, febrile patients may have normal concentrations of C-reactive protein, and discordance between the plasma concentrations of different acute-phase proteins is common. These variations, which indicate that the components of the acute-phase response are individually regulated, may be explained in part by differences in the patterns of production of specific cytokines or their modulators in different pathophysiologic states (Gabay and Kushner, 1999).

Table 1.4 Other Acute-Phase Phenomena.

Neuroendocrine changes
Fever, somnolence, and anorexia
Increased secretion of corticotropin-releasing hormone,
Decreased production of insulin-like growth factor I
Increased adrenal secretion of catecholamines
Hematopoietic changes
Anemia of chronic disease
Leukocytosis
Thrombocytosis
Metabolic changes
Loss of muscle and negative nitrogen balance
Decreased gluconeogenesis
Increased hepatic lipogenesis
Increased lipolysis in adipose tissue
Decraesed lipoprotein lipase activity in muscle and adipose tissue
Cachexia
Hepatic changes
Increased metallothionein, inducible nitric oxide synthase,
tissue inhibitor of metalloproteinase-1
decreased phosphopyruvate carboxykinase activity
hypozincemia, hypoferremia, and hypercupremia
increased plasma retinol and glutathione concentrations

In summary acute-phase response includes stereotyped, coordinated adaptations ranging from behavioral to physiologic changes (Table 1.4), in an attempt from the body to localize and limit the injury and clear tissue debris (Kushner, 1993). Acute phase response includes the hepatic synthesis of large quantities of proteins with various functions, which include binding proteins (opsonins), protease inhibitors, complement factors, apoproteins, fibrinogen, and others.

1.3.7 C-reactive protein and colorectal cancer

C-reactive protein (CRP) is one of the acute phase proteins and is produced almost exclusively by the liver during episodes of acute inflammation. Local inflammation and tissue injury if of sufficient magnitude may signal the liver to produce and release C-reactive protein into the bloodstream (Pasche and Serhan, 2004). Specifically, C-reactive protein is stimulated by pro-inflammatory cytokines, mainly interleukin- 6 (IL-6), this response is enhanced in combination with IL1-β and TNF-α (Gabay and Kushner, 1999). A major function of C-reactive protein, a component of the innate immune system, is its ability to bind phosphocholine and thus recognize some foreign pathogens as well as phospholipid constituents of damaged cells (Volanakis, 1997). It can activate the complement system when bound to one of its ligands and can also bind to phagocytic cells, an observation suggesting that it can initiate the elimination of targeted cells by its interaction with both humoral and cellular effector systems of inflammation (Volanakis, 1997). Other proinflammatory effects of C-reactive protein include the induction of inflammatory cytokines and tissue factor in monocytes (Ballou et al, 1992; Germak et al, 1993). However, it would appear that its net effect is anti-inflammatory (Ahmed et al, 1996; Xia et al, 1997). Such an effect of C-reactive protein may be explained by its ability to prevent the adhesion of neutrophils to endothelial cells by decreasing the surface expression of L-selectin (Zouki et al, 1997), to inhibit the generation of superoxide by neutrophils, and to stimulate the synthesis of interleukin-1-receptor antagonist by mononuclear cells. Therefore, it appears that C-reactive protein has many patho-physiological roles in the inflammatory process.

The biological malignancy of human cancer in general is determined by the net effects of biological malignancy of tumor cells and host factors. Inflammation or presence of

systemic inflammatory response as evidence by raised C-reactive protein is closely related to the host immune responses (Du Clos, 2000). Increased C-reactive protein levels may reflect both inflammatory status and an attempt by the host to suppress tumor formation or growth. On the other hand C-reactive protein stimulates release of more interleukin-6 through activation of complement system (the complement-split products stimulates release of more interleukin-6 by macropghages), which in turn stimulate the synthesis and secretion of more C-reactive protein in the liver, this a completes a positive feedback loop (Kotler, 2000). The release of pro-inflammatory cytokines and growth factors in the presence of the tumour, may promote tumour growth and differentiation (Abramovitch et al, 1999: Balkwill et al, 2001). They also have profound catabolic effects on host metabolism (McMillan et al, 1994: Barber et al, 2000). Interleukin-6 or interleukin-1 type cytokines produced either by the tumour or surrounding cells (Tisdale, 1999) stimulates liver production of acute-phase protein (such as C-reactive protein and fibrinogen) in the fasted and fed state (Barber et al, 2000). The increase in the production of the acute-phase proteins results in an increase in the demand for certain amino acids which, if limited in the diet, may be obtained from the breakdown of skeletal muscle (Preston et al, 1998: McMillan et al, 1998). In these ways the presence and magnitude of a chronic systemic inflammatory response in advanced cancer patients may produce a progressive nutritional and functional decline ultimately resulting in reduced survival.

Recently, Nozoe and co-workers (2000) in cohort of 155 colorectal cancer patients showed that preoperative elevation of serum CRP is related to the reduction of lymphocyte percentages in peripheral blood, and it can be an indicator of impaired immunity in the patients with colorectal cancer. The clinico-pathological characteristic

of tumour (tumour size, the proportions of liver metastases, the lymph node metastases, and the venous invasion) were significantly greater in patients with a preoperative elevation of serum C-reactive protein (Nozoe et al, 2003).

C-reactive protein elevation is considered by many authors as a prognostic factor in patients with operable colorectal cancer. Preoperative C- reactive protein concentration in patients with late-stage colorectal tumours were considerably higher than those in patients with early-stage malignant conditions in most studies (Durdey et al, 1984; Stamatiadis et al, 1992; Nozoe et al, 1998; Nielsen et al, 2000). Moreover, elevated levels of C-reactive protein or interleukin-6, a positive regulatory cytokine for C-reactive protein (Gabay and Kushner, 1999), in patients with colorectal cancer were associated with advanced tumour stage, recurrence and reduced survival (McMillan et al, 1995; Nozoe et al, 1998; Kinoshita et al, 1999; Belluco et al, 2000; Wigmore et al, 2001; McMillan et al, 2001; Chung and Chang, 2003; McMillan et al; 2003). In addition, interleukin-6 levels in colorectal tumor tissue were substantially higher than those in normal tissue (Komoda et al, 1998; Kinoshita et al, 1999). Finally, 2 small case–control studies reported higher C-reactive protein or interleukin-6 levels in patients with colorectal cancer than in controls (Kinoshita et al, 1999; Chung and Chang, 2003). However, in the largest study to date of almost 600 patients undergoing elective surgery for colorectal cancer, Nielsen and his co-worker (2000) reported that an increased pre operative C-reactive protein was a prognostic factor, independent of Dukes' stage.

Therefore, there is increasing evidence that the presence of a systemic inflammatory response, as evidenced by an elevated C-reactive protein concentration, is associated with poorer survival in patients undergoing resection for colorectal cancer. It also

would appear that the systemic inflammatory response promotes tumour growth and dissemination. However, it remains to be determined whether an elevated C-reactive protein concentration reflects a nonspecific inflammatory response secondary to tumor necrosis or local tissue damage or alternatively, whether its development reflects the phenotype and metastatic potential of the tumour.

1.4 Staging for Colorectal Cancer

1.4.1 Dukes' classification

Lochart-Mummery (1927), noted a relationship between prognosis and extension of rectal cancers, suggesting a clinical classification that was later modified to include pathological data (Gordon-Watson and Dukes, 1929). Dukes subsequently changed the criteria, developing a system for the pathological classification of rectal carcinoma (Dukes, 1932). Dukes classified rectal tumours into those not penetrating the bowel wall and without lymph node metastases (A), those penetrating the wall, but still without lymph node metastases (B), and finally tumours with lymph node metastases, regardless of the depth of penetration of the cancer (C). Stage C was modified by Dukes in 1935 (Gabriel et al, 1935), dividing it in CI with lymph node metastases near to the rectal wall and C2 with lymph node metastases near to the ligation of the superior rectal artery.

Kirklin and co-worker (1949) extended the use of Dukes' staging to include the colon, but it has been shown that prognosis may be different in the colon compared with the rectum in patients with the same Dukes' stage (Pihl et al, 1980). Kirklin and co-worker (1949) also changed the meaning of Dukes' A, now being limited to the mucosa, whereas B1 would mean penetrating into the mucosa, but not through the muscularis propria, and B2 penetrating the bowel wall, including the serosal surface in colonic tumours. This change of definition in Dukes' stage A has caused much confusion, because it has been adapted in America but not by Europeans, who still prefer Dukes' A to mean cancers which do not penetrate through the muscularis propria. It must be emphasised that the definition of colorectal cancer includes penetration of the lamina muscularis mucosa, which has not always been pointed out in American literature

(Enker et al, 1979) presenting tumours confined to the mucosa, not mentioning whether penetration of the muscularis mucosae has occurred; it is believed that tumours have to penetrate this layer before they are able to spread to lymph nodes. The conclusion has resulted in over treatment as well as insufficient treatment; it is strongly recommended that tumours not penetrating the lamina muscularis mucosae should be called no more than adenomas with severe dysplasia, and words like carcinoma in situ and intramucosal carcinoma should be avoided (Jass et al, 1987).

Astler and Coller (1954) subdivided Dukes' C into C1 meaning regional lymph node metastases, but tumour limited to the bowel wall and C2 when the tumour penetrated through the bowel wall with lymph node metastases; however, stage C1 only presents a small part of all colorectal cancer (Phillips et al, 1984). In this context, it should be remembered that degree of invasion may vary in different parts of the tumour making it mandatory to look at several sections before deciding whether the muscularis propria has been penetrated (Rubio et al, 1977). Unfortunately, the authors did not add anything to solving the problem of obtaining a uniform terminology for Dukes' stage A. The previous staging was based on examination of resected bowel and adjacent mesentery. Turnbull and co-workers (1967) introduced a clinicopathological staging which has achieved worldwide use; he adapted the staging by Dukes (1932), but added a stage D (Table 1.5), meaning metastases to distant organs; patients with unresectable tumours because of parietal invasion or invasion of neighbouring organs were also placed in stage D. Again, most Europeans have not used the last distinction, but usually they mention whether Dukes' B or C tumour have invaded the abdominal wall or other organs.

It must be realised that survival figures may vary, depending upon the thoroughness with which the pathologist looks for penetration of the bowel wall and lymph node metastases. Also, 5-year survival is sometimes reported after exclusion of immediate postoperative mortality (Whittaker et al, 1976). The large variations in survival figures (Whittaker et al, 1976; Nilsson et al, 1984; Ohman, 1985; Kune et al, 1990) suggest that Dukes' staging is unreliable; however, it is the simplest method today, and it is questionable whether prognostic discrimination will improve by using the TNM system, unless clinical and other than pathological variables are included in the staging procedure (Wood, 1968; UICC, 1978).

The histological grade of the tumour and the number of involved nodes, which are both known to be related to survival (Wolmark et al, 1983; Jass et al, 1987; Quirk et al, 1987; Fisher et al, 1989), are not considered in Dukes' staging. The thoroughness of the pathologist in examining the lymph nodes is also important. Blenkinsopp and co-workers (1981), reviewing pathological assessment of the depth of tumour penetration in 2046 patients from several institution, noted that variation between observers ranged from 5 to 30 per cent. Differences were also noted between centres in the mean number of lymph nodes harvested per specimen and the proportion of cases in which no nodes were identified.

Irrespective of these problems, there are deficiencies in the entire Dukes' classification. Analysis based on a few tissue samples may miss break through of the muscularis propria, such that some patients who die quite soon are misclassified into the good prognosis Dukes' A group. In series of 2037 rectal cancers, Dukes reported a crude 5-

year survival rate of 81.2 per cent for patient with Dukes' A cancer, instead of the near 100 per cent expected if the staging were perfect (Dukes, 1958).

Despite these problems, Dukes' classification remains an extremely powerful prognostic tool. Major series employing multivariate analysis have identified tumour stage as the primary determinant of prognosis in colorectal cancer (Quirke et al, 1987; Griffin et al, 1987; Wiggers et al, 1988).

In conclusion, the deficiency of Dukes' classification is that it fails to take clinical factors and number of involved nodes into account. Modifications have resulted only in confusion over the interpretation of the various classes, particularly stage A. In spite of these problems, the prognostic significance and comparative ease of Dukes' classification ensure that it remains the gold standard against which all other prognostic variables in colorectal cancer be tested.

Table 1.5 Dukes' classification and its impact on outcome in colorectal cancer (Adapted from McArdle and Hole, 2002).

DUKES' STAGE	PROPORTION OF PATIENTS	5-YEAR SURVIVAL
Stage A	7 %	85 - 95 %
Stage B	47 %	70 - 75 %
Stage C	30 %	35 - 45 %
Stage D*	15 %	0 - 5 %

*Dukes' original classification did not include a stage D

1.4.2 TNM Classification:

In an attempt to overcome the problem of Dukes' Classification, the International Union Against Cancer (UICC) proposed a clinical classification in 1966, separating colonic and rectal cancer (Wood, 1968). However, the proposition was withdrawn and tumours were then classified by extent of primary tumour (T), condition of regional lymph nodes (N) and presence or absence of distant metastases (M) at time of diagnosis, including a pretreatment clinical classification (TNM) as well as a post surgical histopathological classification (pTNM) (UICC, 1978; Beahrs, 1989).

The TNM system has undergone six revisions and, in the United States, these changes have been guided by the American Joint Committee on Cancer (AJCC), which was established in 1959 and which has published a succession of revisions of its AJCC Cancer Staging Manual. Until the sixth edition (Greene et al, 2002), the TNM stages for colon cancer had not changed substantially in 35 years. The stages in the first AJCC staging manual, published in 1976 (AJCC, 1976), were virtually identical to those in the fifth edition (Fleming et al, 1997), except for T2 having moved from stage II in the first edition to stage I by the fifth edition. O'Connell and co-workers (2004) reported that the sixth edition of the AJCC Cancer Staging Manual is substantially different from the fifth edition in terms of stages II and III (Figure 1.6&7). In the fifth edition, stage II was composed of (T3, N0, M0) and (T4, N0, M0). In the sixth edition, stage II is divided into two sub stages: IIa, which is (T3, N0, M0), and IIb, which is (T4, N0, M0). This change indicates that IIb patients will, on average, have a worse prognosis than stage IIa patients and a better prognosis than stage IIIa (T1–T2, N1, M0) patients. This finding appear to violate the rules of the stage model upon which the TNM system is based. The use of the colon cancer staging in the sixth edition may result in uncertainty in clinical

trials and potentially incorrect patient therapy. Therefore, until the advent of the seventh edition, clinicians and researchers may not use the sixth edition's colon cancer staging; rather, relying on the previous (fifth) edition.

O'Connell and co-workers (2004) evaluated the new AJCC sixth edition staging system for colon cancer. They found that the new AJCC sixth edition staging system for colon cancer results in better estimates of survival by providing more sub stages than the fifth edition system (Table 1.6). In addition, they demonstrated that other factors may be used to improve colon cancer staging, such as tumour grade and additional stratification of the number of positive lymph nodes. It is unclear why stage IIIa patients appear to have better survival than that of stage IIb, but this finding brings up the important and debated question of whether patients with stage II colon cancer should receive adjuvant therapy. Further studies to determine whether stage IIb patients would benefit from receiving chemotherapy routinely may be indicated.

Table 1.6 Stages as defined by the American Joint Committee on Cancer (AJCC) fifth and sixth edition staging systems (Adapted from O'Connell, 2004).

Staging system	T stage	N stage	M stage
AJCC fifth edition			
I	T1 or T2	N0	M0
II	T3 or T4	N0	M0
III	Any T	N1	M0
IV	Any T	Any N	M1
AJCC sixth edition			
I	T1 or T2	N0	M0
IIa	T3	N0	M0
IIb	T4	N0	M0
IIIa	T1 or T2	N1	M0
IIIb	T3 or T4	N1	M0
IIIc	Any T	N2	M0
IV	Any T	Any N	M1

T1 = tumor invades submucosa; T2 = tumor invades muscularis propria; T3 = tumor invades through the muscularis propria into the subserosa or into nonperitonealized pericolic tissues; T4 = tumor directly invades other organs or structures and/or perforates visceral peritoneum; N0 = no regional lymph node metastasis; N1 = metastasis to one to three regional lymph nodes; N2 = metastasis to four or more regional lymph nodes; M0 = no distant metastasis; M1 = distant metastasis.

Table 1.7 Five-year survival by American Joint Committee on Cancer fifth edition system stages I–IV (Adapted from O'Connell, 2004).

Stage	3-Years Survival (%)	5-Years Survival (%)
I	96.1	93.2
II	89.2	82.5
III	72.7	59.5
IV	17.3	8.1

In summary; the future utility of the TNM staging system depends on its ability to deal with the increase in population screening for cancer, the discovery of new therapies, and the use of the new molecular biomarkers. First, the predictive accuracy of the TNM system depends on patients presenting across the entire chronological range of the disease. Because the system depends on the sequential progression of tumors for its predictive accuracy, anything that reduces the time of disease progression will reduces its accuracy. The early detection of disease because of screening results in a shift in populating the stages to earlier disease so that the majority of patients are in stage I or at most stages I and II. This stage compression reduces the accuracy of the TNM staging system. Adding to the stage compression problem are the trends of very small surgical specimens and to neo-adjuvant therapy. Both these changes in the clinical approach to disease further reduce the size of tumors, the detection of nodal metastases, and the accurate grading of tumors due to changes in histology.

Furthermore, the viability of the temporal model of TNM staging has been questioned by observational studies of breast cancer; for example, some patients with small tumors and no evidence of lymph node involvement die of their disease earlier than patients who present with more advanced disease (Fisher et al, 1978). Second, the TNM staging system does not take into account neo-adjuvant therapy, anti-hormonal therapy, chemotherapy, or the new, targeted, monoclonal molecular therapies. Thus, changes in survival related to these therapies are not reflected in the TNM stages. For the TNM system to incorporate new therapies into its stages, it must stratify by therapy (Burke et al, 1998). If staging does not incorporate new therapies, it will not be useful in predicting patient outcomes beyond what is predicted to happen if the patient undergoes only surgery. Third, the TNM staging system has difficulty dealing with continuous

biomarkers and adding new biomarkers. It has been reported that a computer-based statistical program that allows the T and N variables to remain continuous appear to give more accurate prediction of 5- and 10-year disease-specific survival for breast and colon cancer than the TNM staging system (Burke et al, 1997). Carcinogenesis is not defined by what stage the patient is in at detection but rather by the molecular (genomic) characteristics of the tumor and the host reaction. Biological determinism takes the view that the anatomic location of the disease at detection is more related to our methods of detection than to the tumor itself. Thus, all patients are at risk of metastatic disease; some are further along a biological metastatic pathway at detection than others. In this light, treatment should be driven by the molecular biology of the tumor or host and not the tumor's location at detection.

1.4.3 Jass's classification

Although Dukes' classification was extremely helpful and easy to use, it failed to employ potentially important clinicopathological variables, while the TNM system, containing some of these variables, was too complex for routine use. The advent of multivariate survival analysis allowed Jass and co-workers (1986) to develop a system based on tumour stage and series of pathological variables related to histological grade. Jass and co-workers (1987) identified four independent variables, which have an influence on clinical outcome. These factors were selected by means of multivariate regression analysis of 379 specimens of patients who underwent curative surgery for rectal cancer. These factors are: the character of invasive margin, the presence of lymphocytic infiltration, local tumour spread and the number of lymph nodes with metastatic tumour (Figure 1.9). Histopathological variables include:

Invasive margin

Cancers were described as expanding when invasive margin was pushing or reasonably well circumscribed, and infiltrating when the tumour invaded in a diffuse manner with widespread penetration of normal tissues.

Lymphocytic infiltration

This was regarded as conspicuous when there was a distinctive mantle or cap at the invasive margin of the growth, consisting of lymphocytes and other inflammatory cells.

Local tumour spread

This was described as none (limited to the rectal wall), slight to moderate (spread beyond the rectal wall), and extensive. Extent of spread beyond the bowel wall was based on a subjective assessment made at the time of the original dissection.

Lymph node metastasis

This was grouped as none, 1-4 nodes and more than 4 nodes involved.

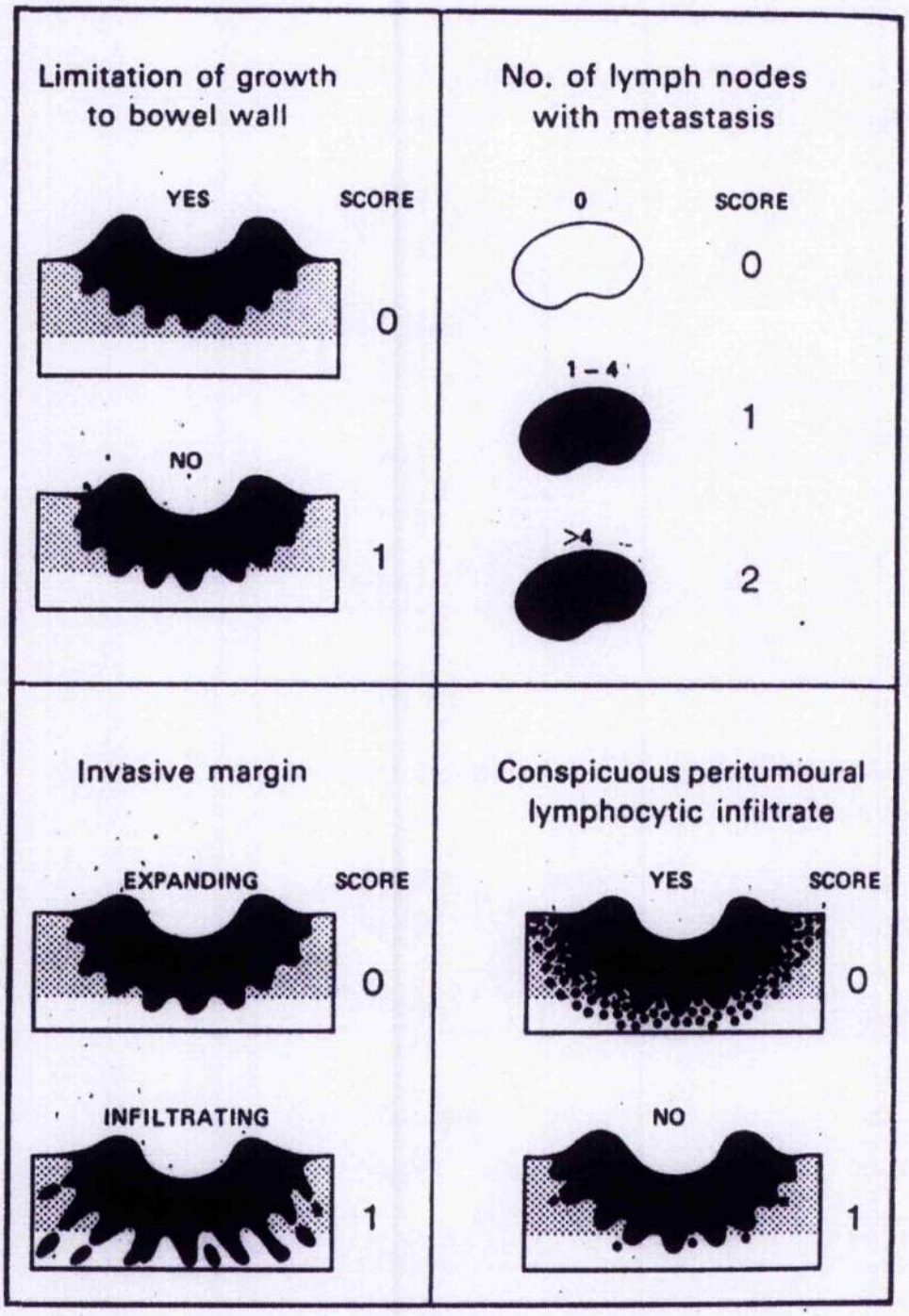
Then these divided into groups, as following (adapted from Jass, 1986):

:

GROUP	TOTAL SCORE	PROGNOSIS (5 YEARS SURVIVAL)
I	0 - 1	96%
II	2	85%
III	3	67%
IV	4 - 5	27%

This classification was claimed to be superior to that of Dukes' classification (Jass et al, 1986; Jass et al, 1987; Jass et al, 1990). Although all of the grade-related variables investigated by Jass and co-workers (1986) correlated with survival on univariate analysis, only three remained important after Cox's regression analysis. After correction for Dukes' classification, the degree of lymphocytic infiltration was the only grade-related variable that remained significantly related to survival.

Figure 1.9 Scoring system for pathological variables (adapted from Jass, 1987).



The new prognostic classification was superior to that of Dukes because it placed twice as many patients into groups that provided a confident predication of clinical outcome. As well as offering prognostic guidance it was suggested that the system would be of value in selecting patients (group IV) who might receive the most benefit from adjuvant therapy aimed at eradicating occult disease (Jass, 1987).

The success of any system of classification depends upon the care devoted to the collection of the data upon which it is based. Four pathological variables are used in the present Jass system. Two of these are subjective (character of the invasive margin and presence of a peritumoural lymphocytic infiltration), but can be assessed rapidly and with high levels intra-observer agreement (Jass, 1986). Spread within or beyond the bowel wall can be evaluated readily. The subjective division of extramural spread (defined as spread beyond the muscularis propria irrespective of the presence or absence of peritoneum) into slight or moderate versus extensive did not improve the model (Jass, 1987). The most important variable is lymph node metastasis, but unfortunately the systematic removal and examination of lymph nodes is the most time-consuming aspect of pathological examination.

Other variables (tumour type, grade of differentiation, venous invasion, and involvement of the apical lymph node) provide no clinically important improvement to Jass prognostic model. However, to be of clinical relevance a system must be reproducible as well as of prognostic significance. Jass's classification relies on the pathologist interpreting visual images into verbal categories and is potentially subjective. Jass and co-worker (1987) initially assessed intra-observer variation by one specialist pathologist and subsequently reported fair to excellent level of inter-observer

agreement for tumour type, differentiation, growth pattern and lymphocytic infiltration (Shepherd et al, 1989)

These assessments were performed by specialist pathologists from a centre dealing almost entirely with colorectal cancer. Reporting using non-specialist general pathologists, which may be more relevant to the majority of pathology departments nationwide, has suggested that observer consistency of many of the components of Jass's classification is poor (Dundas et al, 1988; Deans, 1991). In particular, the intra-observer and inter-observer variation of lymphocytic infiltration in some series was little better than chance (Dundas et al, 1988; Deans, 1991).

Invasive growth pattern and lymphocytic infiltration are the most subjective element of the Jass classification and their usefulness has been the subject of debate (Fisher et al, 1989; Harrison et al, 1994; Deans et al, 1994). For example, other workers have found that lymphocytic infiltration failed to be an independent prognostic variable because of the degree of subjectivity involved in its assessment (Dundas et al, 1988; Shepherd et al, 1989; Fisher et al, 1989). Secco and co-workers (1990) found that there was a significant relation between lymphocytic infiltration and survival in colorectal cancer. However, they failed to demonstrate a significant difference in survival between Jass's grades II and III.

On the other hand, several multivariate studies have shown that peritumoral lymphocytic infiltration (Jass, 1987; Halvorsen, 1989; Secco et al, 1990; Jass, 1990; Kubota et al, 1992; Di Giorgio, 1992; Deans et al, 1994) and/or tumour growth pattern (Jass, 1987; Quirke, 1987; Halvorsen, 1989; Shepherd, 1989; Jass, 1990; Kubota et al,

1992; Gagliardi et al, 1995) serve as independent prognostic factors in large bowel cancer.

Reports employing both Dukes and Jass classifications have suggested that the former was more significantly related to survival on multivariate analysis (Deans, 1991; Fisher et al, 1989). For example, Fisher and co-workers (1989) compared the prognostic values of the Dukes and Jass's systems in 722 patients with rectal cancer. The Jass system allowed for only two major prognostic groups, whereas five were noted by Dukes' method. Both Fisher (1989) and Deans (1994) and their co-workers reached the same conclusion; that is that Dukes' classification is of greater prognostic value and more reproducible than the components of Jass' classification.

Recently De Quay et al (1999) in a cohort observational study of 108 consecutive patients undergoing potentially curative surgery for Dukes' B colorectal carcinoma, found the nature of the invasive margin of the tumour (as a component of Jass's classification) allowed these patients to be separated into different prognostic groups. This means that a group of patients with a Dukes' B tumours whose prognosis is similar to that of those with a Dukes' C tumour can be identified, the nature of the margin of invasion being used to classify a greater proportion of patients.

Because the Jass's classification is not reproducible, not simple and requires a certain degree of pathological experience as compared to Dukes' and TNM classifications, it is not routine in hospital laboratories. However, an interesting feature of the Jass classification is that it is the only pathological system that includes a host-response i.e.

lymphocytic infiltration, which may be an important factor in the defence against tumours.

As none of the above systems used in the classification of colorectal cancers are able to identify precisely those patients, who at the time of curative resection have occult metastases and who in turn would benefit most from adjuvant chemotherapy, the search will continue to avoid giving toxic treatment to patients who may or may not develop a recurrence and to cut unnecessary costs of follow up.

1.5 Management of Colorectal Cancer

1.5.1 Clinical Features

The mode of presentation of a colorectal cancer depends on several factors. Broadly speaking there are two categories of presentation, emergency or elective. In general, the more distal the lesion, the more obstructive are the symptoms. The reason is that the left colon has a narrower calibre than the right and its content tends to be less fluid (Aldridge et al, 1986). Usually patients present with clinical features of the primary tumour, but occasionally they may present with symptoms related to metastases.

Change in bowel habit is probably the most common symptom, particularly with left sided lesions (Aldridge et al, 1986). Central or lower abdominal colic is a frequent complaint. Bleeding may be obvious or occult; the blood may vary in hue from bright red to black. The more proximal the lesion, the more altered will the blood become.

Bleeding is the most common symptom of rectal lesion, and is frequently ignored by the patient; the blood is often red, but not bright red. It may be mixed in with the stool or passed separately (Goulston, 1986). The passage of mucus is common; it may be separated from the stool or mixed with it. The patient often describes the symptoms as `passing slime`. A patient with a rectal lesion frequently has the urge to defecate but on going to the lavatory passes only blood and mucus. Such `tenesmus` is often most acute in early morning and occurs soon after the patient rises from bed (Morris, & Wood, 2000)

1.5.2 Diagnosis of colorectal cancer

Physical Examination

A full clinical examination is essential in all cases of suspected colorectal carcinoma. The tumour itself may be palpable; right-sided lesions are stated to be more often palpable than left-sided ones (Goligher, 1984).

After general and abdominal examination it is necessary to proceed with digital examination of the rectum. Approximately 75% of carcinomas of the lower two-thirds of the rectum (0—12 cm from anal verge) should be palpable per rectum (Williams et al, 1985).

Proctoscopy and sigmoidoscopy

All patients who on rectal examination have a palpable rectal carcinoma need further evaluation to obtain histology to confirm the diagnosis and to establish whether other lesions in colon are present.

Flexible sigmoidoscopy has the important advantages that it can be passed to a higher level in the colon and the view is far superior to the rigid instrument. For example, McCallum and co-workers (1984) examined 1015 symptomatic patients, ages 20-89 years, using flexible sigmoidoscopy. Eighty five neoplastic lesions were identified in 78 patients. Among patients over the age of 60 years, 3.3% had carcinoma compared with 0.8% of patients younger than 60 years. Of more importance, over 50% of the neoplastic lesions were detected more than 20 cm from the anal verge and were unlikely to have been detected using a rigid instrument. Similar results were reported by Reynolds and co-workers (1983).

It must be stressed that neither barium enema nor colonoscopy alone are adequate investigations to rule out rectal carcinoma and therefore digital rectal examination and sigmoidoscopy must also be undertaken (Keighley & Williams, 1999)

Barium enema examination & Colonoscopy

Double contrast barium enema is used routinely in investigation of colorectal cancer patients. The tumour may cause a stricture either of a `string` or `napkin ring` type. The string type is quite short, usually about 2 cm in length, whereas the napkin ring is up to 8 cm. There is `shouldering` of the growth giving the appearance of an `apple core` deformity, the latter term being sometimes used describe this type of stricture (Keighley & Williams, 1999).

In many units it is still the policy to perform a double-contrast barium enema if digital examination and proctosigmoidoscopy are normal. However, even in the most expert hands it is recognized that radiology can be misleading (Brady et al, 1994).

The errors are usually due to lesions within the caecum or sigmoid colon. For example, Boulos and co-workers (1984) reported a series of 65 patients who all had a barium enema followed by colonoscopy. Of the 46 patients in whom the radiograph showed apparently straightforward diverticular disease, eight (17%) had polyps and three (6%) were found to have a carcinoma. These findings have also been confirmed to some extent by Farrands et al (1983) who, although not using full colonoscopy, found that in 84 patients with polyps or cancer submitted to both flexible sigmoidoscopy and double-

contrast barium enema, there was a miss rate of 25% in the sigmoid colon on barium enema alone, mainly due to coexisting diverticular disease.

Durdey et al (1987) suggested that full colonoscopy should be the first investigation following proctosigmoidoscopy. However, many clinicians believe that is unwise since they consider that colonoscopy carries significantly more risk than barium enema (William et al, 1985). Air leaks are sometimes reported, presumably owing to the high pressure that may be generated by endoscopy, particularly when the tip impinges on the mucosa in a closed loop (Ehrlich et al, 1984; Humphreys et al, 1984). Furthermore a full colonoscopy is not always possible even with modern instrumentation. Even the most skilled operators fail in approximately 10% of patients and the less experienced will naturally be less successful (MacCarty, 1992). More recently, with increasing subspecialisation colonoscopy has became safer and there is a view that it might be used as part of the screening programme for colorectal cancer.

1.5.3 Surgical Treatment of Colorectal Cancer

Principles of Surgery for colorectal cancer

Surgery remains the mainstay of treatment for colorectal cancer. The principles of surgery depend to some extent on whether the operation is being performed in the belief that cure is possible (radical) or whether it is considered a palliative procedure. A radical procedure requires that the tumour be removed with an adequate margin of normal colon together with the associated vascular pedicle and as many of the corresponding lymph nodes as possible. A palliative procedure can be a limited excision of the tumour, a bypass or a defunctioning procedure, each of which is designed to alleviate the patient's symptoms (Keighley & Williams, 1999). Following excision of the tumour-bearing segment of bowel, gastrointestinal continuity is normally restored by anastomosis between the proximal and distal bowel; the exact technique varies according to the individual surgeon's preference. No matter which procedure is performed it should, if possible, be carried out on a well-prepared bowel and with the minimum amount of spillage. Tissues should be handled with care, haemostasis must be absolute and apposition of tissue must be accurate. There is no room for complacency in this type of surgery since leakage from a bowel anastomosis is a potentially lethal complication. (Keighley and Williams, 1999).

For tumours of the right colon, that is, the caecum, ascending colon, hepatic flexure and proximal half of the transverse colon—the ileocolic artery, right colic and the right branch of the middle colic artery are usually divided, and resection of the distal 10 cm of ileum, the caecum, ascending colon and proximal one-third of the transverse colon is carried out. This is the standard right hemicolectomy.

For all tumours of the left colon, which includes those of the distal half of the transverse colon, the splenic flexure and the descending and sigmoid colon, the inferior mesenteric artery should be divided as close to the aorta as possible and the proximal colon anastomosed to the rectum (Williams et al, 1985). Such anastomoses depend on an adequate blood supply from the middle colic artery via an intact marginal artery. In infirm patients a more limited resection may be appropriate.

There are three requirements regarding the elective surgical treatment of rectal cancer: (1) adequate locoregional clearance, (2) minimal complications and (3) satisfactory function. Of these, the first is by far the most important, since local recurrence inevitably results in death in almost all these patients (Welch, 1974).

Abdominoperineal resection (APR) is generally indicated for lesion of the lower one third of the rectum or for higher lesion in the presence of an incompetent anal sphincter. It may also be indicated in morbidly obese patients, particularly males with a narrow pelvis, in patients with significant anal sphincter weakness, and with bulky or poorly differentiated lesions located less than 2 cm from the dentate line (Pricolo, 2001). APR includes complete removal of the distal sigmoid, rectum, and anal sphincteric complex through combined abdominal and perineal approaches, with creation of a permanent colostomy. Until recently, this operation was considered the standard for rectal cancers located less than 5 cm from the dentate line (Kirwan et al, 1989).

Nowadays, a sphincter-saving procedure is possible in the majority of rectal cancers. Many studies have shown that if a margin of 2.5 cm of normal rectum below the distal border of the tumour is achieved by anterior resection, there is no compromise of

survival or local clearance compared with those of abdominoperineal excision (Pollet et al, 1983; Williams et al, 1984; Phillips et al, 1984). This means that most tumours 6 cm or more from the anal verge, and therefore within 2 or 3 cm above the anorectal junction, are suitable for restorative resection. Also the availability of endoluminal circular stapling devices and the often significant reduction of size in bulky distal rectal cancer with preoperative radiation therapy have made sphincter preservation the rule rather than the exception for cancer of the distal one third of the rectum (Pricolo, 2001).

The concept of total mesorectal excision (TME) has also had a significant impact on treatment of distal rectal cancer over the past two decades. Heald and co-workers (1982) deserve credit for attracting the attention of the surgical community to the need for complete excision of the mesorectum in all pelvic resection for rectal cancer. Heald and co-workers (1982) observed microscopic deposits in a lymphatic vessel 4 cm below the edge of a rectal cancer with no nodal involvement, and further studies of the distal mesorectum suggest that these deposits are by no means rare.

The technique of mesorectal excision involves the development of an avascular plane between visceral structures (rectum and mesorectum) and the autonomic nerve plexuses by sharp dissection, thereby protecting the pelvic wall, genito-urinary structures, presacal nerves, and parasympathetic roots. Heald (1988) described this avascular plane as "Holy plane". Macfarlane (1993) independently, analysed the data produced by Heald and his co-workers (1986) with prospective follow-up extended over a 10 years interval. The local recurrence rate in the curative resection subgroup was less than 4%. Indeed, most series from Europe and the United States have reported local recurrence rates less than 10% and satisfactory long-term survival with total mesorectal excision

alone (Arbman et al, 1996; Aitken, 1996; Arenas et al, 1998; Martling et al, 2000). These results are better than that in the surgery-alone arm in most adjuvant therapy trials (Fisher et al, 1988; Gerard et al, 1988; Krook et al, 1991). More recent studies have confirmed that nodal metastases may be present in the mesorectum distal to the primary tumour, in approximately 20% of patients (Scott et al, 1995; Reynolds et al, 1996; Hida et al, 1997).

In summary, surgical technique remains an extremely important factor in determining long term survival of patients with colorectal cancer. With increasing sub-specialisation of colorectal surgery it is likely that there will further improvements in outcome following potentially curative resection for colorectal cancer.

CHAPTER TWO

2.1 Hypothesis

There is now good evidence that the presence of a systemic inflammatory response plays a pivotal role in defining outcome in variety of sold tumours, including colorectal cancer. For example, the presence of a systemic inflammatory response is associated with increased recurrence and decreased survival independent of Dukes stage, in patients undergoing surgery for colorectal cancer.

The interrelationship between tumour and host factors and disease progression in patients with colorectal cancer is not clear. The presence of the tumour cells will activate the innate immune system as part of the host reaction to the presence of tumour. The attraction and activation of various inflammatory cells at or near the tumour site will lead to the release of various cytokines. Some will act locally on other inflammatory cells while others will find their way in to the circulation and activate the systemic inflammatory response. The release of IL-6 into the circulation will activate the release of the acute phase proteins in the liver. The adaptive immune system will be activated due to the presence of antigens in the tumour. These tumour specific antigens will activate the humoral immune system and add to the inflammatory response.

It is recognised that the systemic inflammatory response is primarily driven by the local and systemic release of the pro-inflammatory cytokines, in particular interleukin-6. However, the source of increased circulating interleukin-6 is not clear. Clearly, the increase in circulating interleukin-6 could be due to increased production by the tumour or secondary to the release of tumour products which stimulate host production of interleukin-6. Recently, the source of interleukin-6 has been localised to the tumour and

this would be consistent with the observation that circulating concentrations of interleukin-6 and C-reactive protein are related to tumour size and that concentrations fall after resection of the primary tumour. However, it is not clear whether the increased production of interleukin-6 by the tumour arises from the tumour cells or the associated inflammatory infiltrate.

It has been hypothesized that in patients with cancer, either the tumour cells or the host cells or a combination of the two are responsible for the production of the pro-inflammatory cytokines that induce the systemic inflammatory response. These cytokines will trigger the change in protein metabolism, characterised by increased rates of synthesis of acute phase proteins using amino acids from skeletal muscle, which may contribute to the muscle wasting seen in cancer cachexia.

The pro-inflammatory cytokines mainly IL-6 trigger the release of C-reactive protein which is a part of the acute phase proteins. Rising acute phase reaction proteins are usually a sign of disturbance of the host-tumour relationship with the survival probability tending to decrease as the levels of acute phase reaction proteins rise. Such an intense reaction, will promote the tumour growth locally and affect the patient in general through cachexia.

The aim of the proposed study was to examine the relationship between the systemic inflammatory response, tumour inflammatory infiltrate and tumour proliferative index in patients with primary operable colorectal cancer. These factors will also be related to survival.

2.2 Aims

The aims of this project were to:

1. Examine the prognostic value of elevated circulating C-reactive protein concentrations and its combination with Dukes stage to form a new prognostic score in patients undergoing apparently curative resection for colorectal cancer.

2. Examine the relationships between the systemic inflammatory response, tumour lymphocytic infiltration, tumour proliferative activity and cancer survival in patients with primary operable colorectal cancer.

CHAPTER THREE

3.1 Patients

Patients with histologically proven colorectal cancer who, on the basis of pre-operative imaging and the surgeons' assessment at operation, were considered to have undergone potentially curative resection for Dukes B and C colorectal cancer between January 1997 and August 2001 in a single surgical unit at Glasgow Royal Infirmary were included in the study. Those patients who underwent emergency surgery or had pre-operative radiotherapy or subsequently died within 30 days of surgery. Also those patients who showed clinical evidence of infection or other inflammatory conditions were excluded from the study. The tumours were staged using conventional Dukes' classification (Dukes and Bussey, 1958). All patients were followed-up at a colorectal cancer clinic.

The study was approved by the ethics committee of the Royal Infirmary, Glasgow.

3.2 C-reactive protein concentration

Prior to surgery, blood was obtained for routine laboratory measurement of C-reactive protein. Based on work both in apparently healthy elderly individuals (O'Reilly et al, 2006) and in intestinal cancer patients (O'Gorman et al., 2000) an elevated C-reactive protein concentration (>10mg/l) was considered to indicate a significant systemic inflammatory response since less than 1% of apparently healthy elderly individuals and only a proportion of cancer patient have a C-reactive protein concentration in this range. Previously, a variety of cut-offs for C-reactive protein has been used in cancer, for example greater than 5 mg/l in advanced renal cancer (Blay et al., 1992) or 5mg/l in primary operable colorectal cancer (Nozoe et al., 1998). It has been shown that a cut-off of greater than 10 mg/l is superior to either of these cut-offs (Bromwich et al., 2004;

O'Gorman et al., 2000). Clearly, standardizing this cut-off will enable comparison of results between studies and substantiate the importance of the measurement of C-reactive protein in patients with cancer.

C-reactive protein was measured by fluorescence polarization immunoassay using a TDX analyzer and reagents obtained from Abbott Laboratories (Abbott Park, IL). The limit of detection of the assay is a C-reactive protein concentration of >5 mg/l.

3.3 Immunohistochemical staining of CD4+ and CD8+ lymphocytes

The slides for this study were cut from the blocks of the primary tumour taken at the time of the surgical resection. All tissues were fixed in 10% buffered formalin and embedded in paraffin wax. One representative block of tumour was selected for each patient. Sections (4μm) were cut and mounted on slides coated with aminopropyltriethoxysilane.

Sections were then immunostained using a streptavidin biotin technique. The primary antibody for CD4 was mouse monoclonal (Vector, Peterborough, UK) and that for CD8 was mouse monoclonal (Dako, Cambridgeshire, UK). Sections were dewaxed and rehydrated.

Endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide for 10 minutes. Antigen retrieval for CD8 was performed by microwaving in 1 mM EDTA buffer, pH 8, for 5 minutes at full pressure in a plastic pressure cooker in a microwave oven. Antigen retrieval for CD4 was achieved by immersing the sections in high pH buffer (9.9) from Dako for 75 minutes at 99 ^{O}C.

The sections were then incubated with the primary antibodies at dilutions of 1/50 (CD4) and 1/100 (CD8) for 30 minutes at room temperature. The Detection system was Vector laboratories ABC "ELITE" kit (Vector laboratories LTD, Peterborough, UK), and was used as per kit instructions.

Sections were then incubated in 3'3'diaminobenzidine for 10 minutes at room temperature and then in 0.5% copper sulphate in normal saline. Nuclei were then counterstained with haematoxylin, dehydrated, cleared and mounted with synthetic resin.

3.4 Morphometry of CD4+ and CD8+ lymphocytes

The method selected for quantitative analysis of the lymphoid infiltrates was point counting (Anderson and Dunnill, 1965) using a random sampling technique. With this method, the volume occupied by any given component - volume density (V_V) is expressed as a percentage of the total volume of the tissue. In the main study, the volumes of CD4 and CD8 immunopositive cells were calculated as percentage of the total tumour volume. A 100 point ocular grid was used at x 400 magnification and 30 fields were counted per case for each antibody. Only fields containing tumour tissues were counted (Figure 2.1 & Figure 2.2). Any normal tissue on the slide was excluded from the analysis. The final method was designed on the basis of a pilot study.

The volume density (V_V) of CD4 and CD8 positive lymphocytes was calculated for each tumour including tumour nest. A summation average graph was plotted for both CD4 and CD8. The volume density of CD4 and CD8 reached a plateau after 25 to 30

fields. These findings formed the basis for the method of the main study, thirty fields being selected as appropriate for counting. It was also shown that CD4 plus CD8 counts approximately equalled CD3 (Ali et al, 2004). In the full study, therefore, only CD4 and CD8 figures were calculated. All cases were counted by the author. For the purpose of assessing inter-observer reproducibility, a second observer (P McArdle) independently measured CD4+ and CD8+ volume density in a large subgroup of cases. The observers were blinded to the clinical outcome of the patient.

As shown in Table 3.1 and Figures 3.5 and 3.6, there was close agreement between the two observers for measurements of the volume density of both CD4+ and CD8+ lymphocytes. Both the conventional (Pearson) and the intra-class correlation coefficients were greater than 0.90. Differences between observers were statistically significant for CD4+, but the mean difference between observers was less than 0.1% volume density. Differences for CD8+ were statistically insignificant. As shown by the limits of agreement in the Bland-Altman plots, more than 95% of independent measurements of the volume density of either type of lymphocyte would be expected to lie within less than one percentage point of each other.

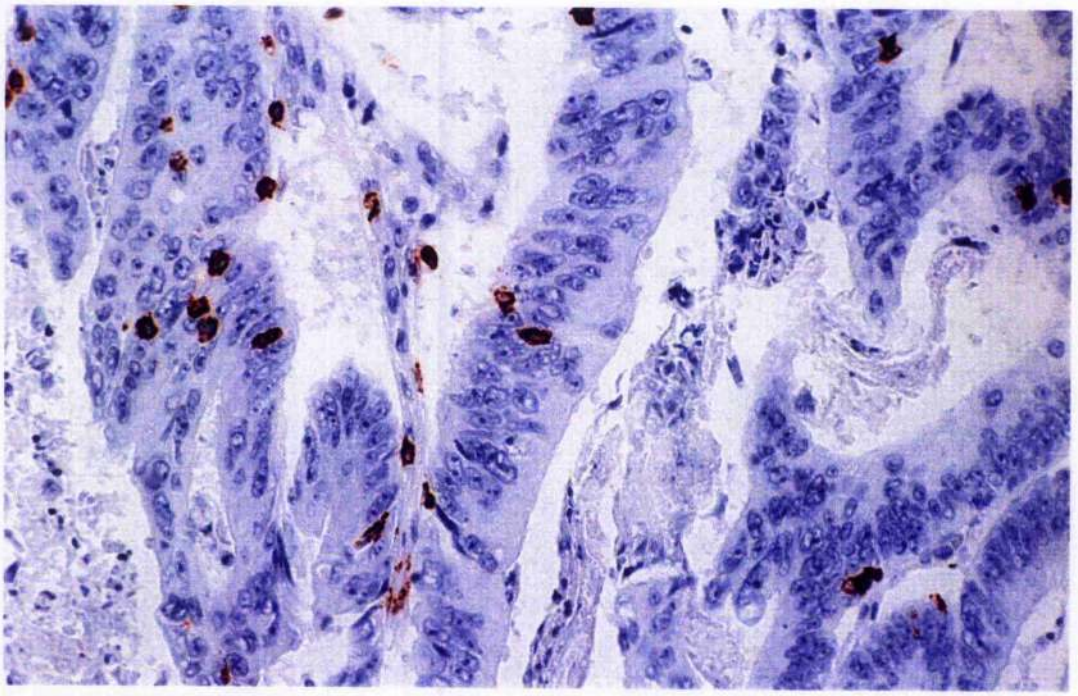


Figure 3.1 Immunohistochemical staining of CD8+ antigen in the tumour tissue.

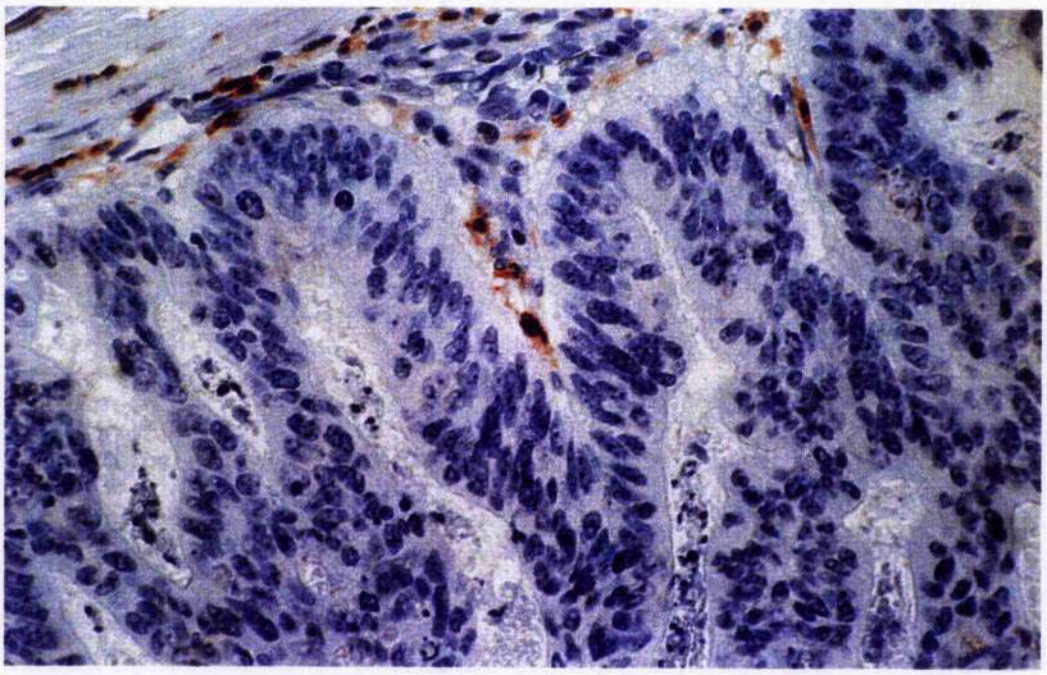


Figure 3.2 Immunohistochemical staining of CD4+ antigen in tumour tissue.

3.5 Immunohistochemical staining of Ki-67 antigen

Sections were immunostained using the ChemMate Dako Envision method (Dako, Cambridgeshire, UK). The primary antibody for Ki-67 was mouse monoclonal antibody (Dako). Sections were dewaxed and rehydrated. Antigen retrieval for Ki-67 was performed by a pressure cooker microwave technique using 1 mM EDTA buffer, pH 8, for 5 minutes.

After antigen retrieval the sections were placed on the Dako Autostainer. In the autostainer the sections were incubated with blocking serum (5% normal goat serum in Tris Buffered Saline (TBS) pH 7.6) for 20 minutes then primary antibodiy at a dilution of 1/200 for 30 minutes at room temperature. Sections were then rinsed in TBS and endogenous peroxidase was blocked by incubation in ChemMate Dako Peroxidase blocking solution for 5 minutes. ChemMate Dako Envision was applied for 30 minutes, sections were again rinsed in TSB. Sections were then incubated in 3'3'diaminobenzidine for 10 minutes at room temperature. Sections were then removed from the machine and the nuclei were then counterstained with haematoxylin. Copper enhancement was done for 5 minutes then the sections were dehydrated, cleared and mounted with synthetic resin.

3.6 Morphometry Ki-67 antigen

The percentages of Ki-67-reactive tumour cells (Figure 3.3) were evaluated with a light microscope (x400 magnification) by scoring a minimum of 1000 tumour cells in randomly selected fields (Ki-67 labelling index). Assessment of Ki-67-reactive tumour cells was carried out blindly by two independent observers (K Canna & M Hilmy) for

sixty cases, counting every case twice. All cases were counted by K Canna and these Ki-67 labelling indexes were taken into the final analysis.

As shown in Figure 3.7, there was reasonable agreement between the two observers for measurement of the Ki-67 labelling index. The mean labelling index was 69% and 64% for the first and second observers respectively, and the difference between observers was statistically significant (mean 5%, 95% confidence interval 3% - 8%, $p<0.001$). As shown by the limits of agreement in the Bland-Altman plot, individual differences between observers could range up to 25%. However, the intra-class correlation coefficient was 0.84, which is within the range conventionally classed as excellent.

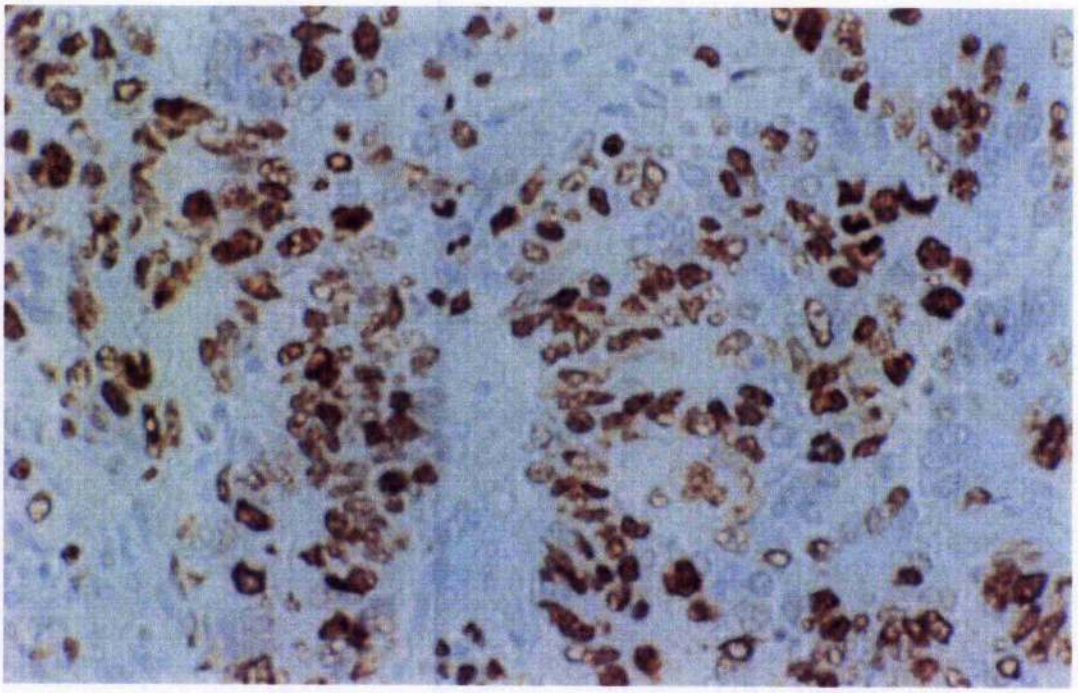


Figure 3. 3 Immunohistochemical staining of Ki-67 antigen in tumour tissue.

3.7 Immunohistochemical staining of interleukin-6

Sections were immunostained using the ChemMate Dako Envision method (Dako, Cambridgeshire, UK). The primary antibody raised against interleukin-6 was a rabbit polyclonal antibody (Abcam, Cambridgeshire, UK) was evaluated as previously described (Kinoshita et al., 1999).

The sections were dewaxed and rehydrated. Antigen retrieval for Il-6 was performed by microwaving with a citrate solution (pH 6) for 5 minutes in a plastic pressure cooker in a microwave oven.

After antigen retrieval the sections were placed on the Dako Autostainer. In the Autostainer the sections were incubated with blocking serum (5 % normal goat serum in Tris Buffered Saline (TBS; pH 7.6). The sections were drained of normal goat serum which was replaced with IL-6 antibody at various dilutions (1:10 - 1:1000) for 30 minutes or overnight at room temperature. Sections were then rinsed in TSB and endogenous peroxidase was blocked by incubation in ChemMate Dako Peroxidase blocking solution for 5 minutes.

ChemMate Dako Envision was applied for 30 minutes; sections were again rinsed in TSB. Section were then incubated in 3`3` diaminobenzidine for 10 minutes at room temperature. Sections were then removed from the machine and nuclei were then counterstained with haematoxylin. Copper enhancement was carried out for 5 minutes and the sections were dehydrated, cleared and mounted with synthetic resin.

The same method was used again with the same rabbit polyclonal IL-6 antibody but different methods of antigen retrieval (EDTA buffer, PH 8 in a plastic pressure cooker in a microwave oven) and various dilutions of the IL-6 antibody. There was however deep background staining with all variations of technique using the rabbit polyclonal IL-6 antibody, which precluded accurate counting and scoring of IL-6 positive cells (Figure 3.4a & b).

The sections were also immunostained using Vector laboratories ABC "ELITE" kit (Vector laboratories LTD, Peterborough, UK), which was used as per kit instructions. The primary antibody raised against interleukin-6 was a goat polyclonal antibody (R&D system, Minneapolis, MN), also using different dilution of the goat polyclonal IL-6 antibody, incubated for 30 minutes and also overnight at room temperature and different methods of antigen retrieval (EDTA buffer, pH 8, or citrate solution, pH 6 in a plastic pressure cooker in a microwave oven). Suitable negative and positive control material were used. The same problem of deep background staining was encountered again (Figure 3.4c & d).

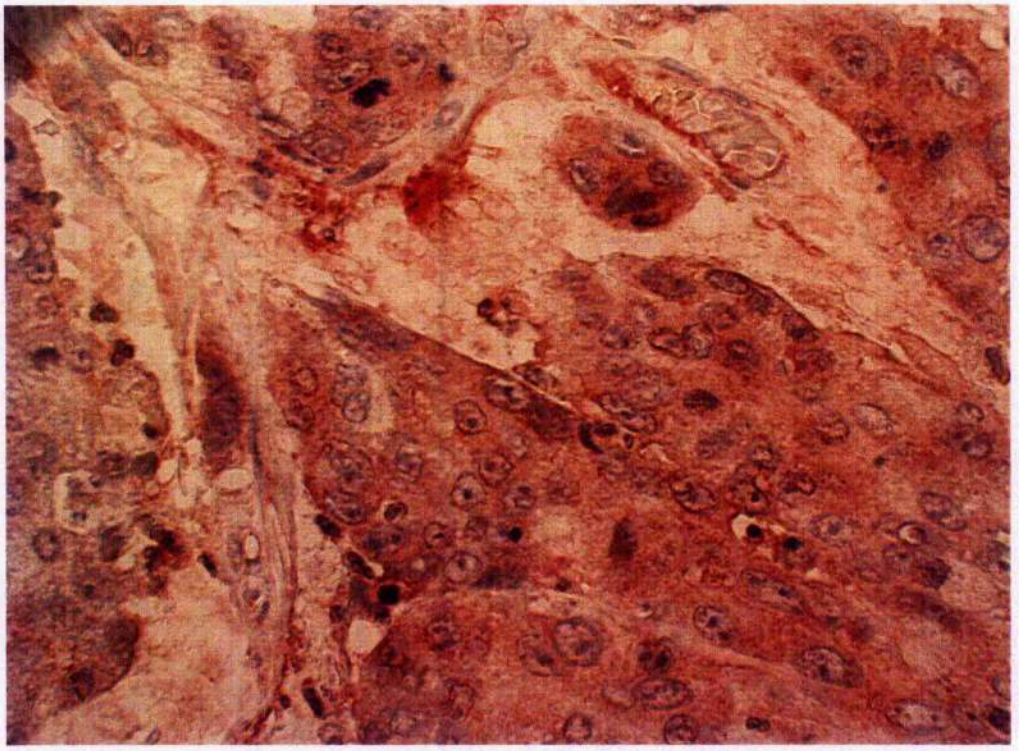


Figure 3.4 (a) Immunohistochemical staining of IL-6 antigen in tumour tissue, deep background staining. (Rabbit IL-6 polyclonal antibody (citrate)).

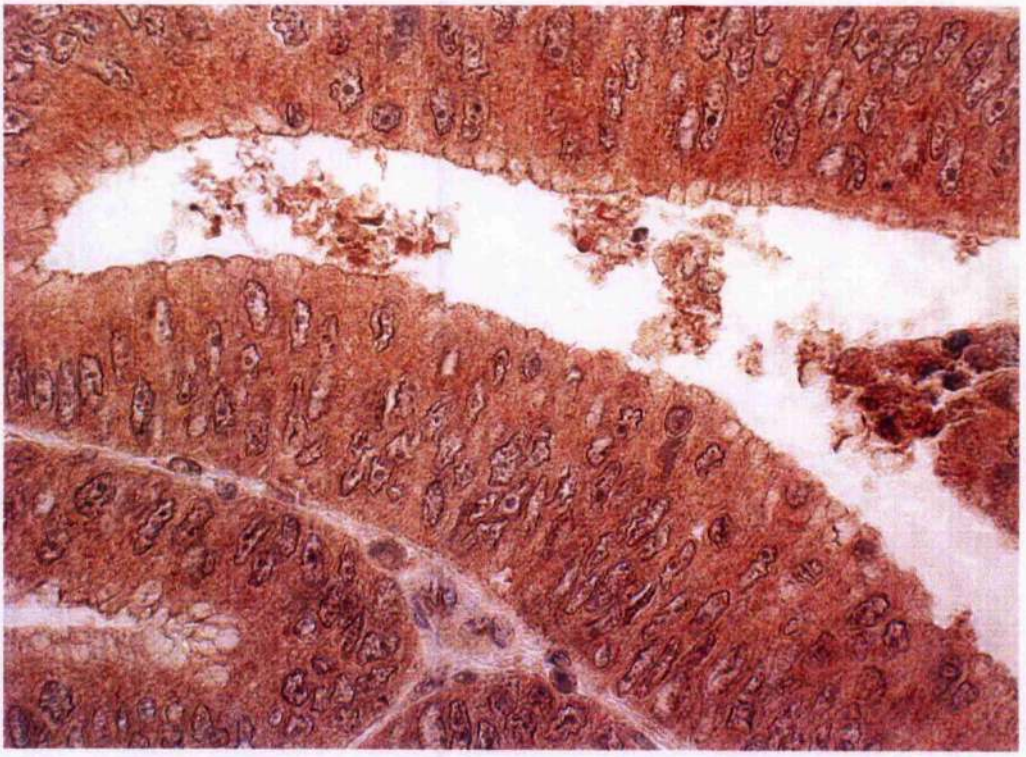


Figure 3.4 (b) Immunohistochemical staining of IL-6 antigen in tumour tissue (Rabbit IL-6 polyclonal antibody (EDTA)).

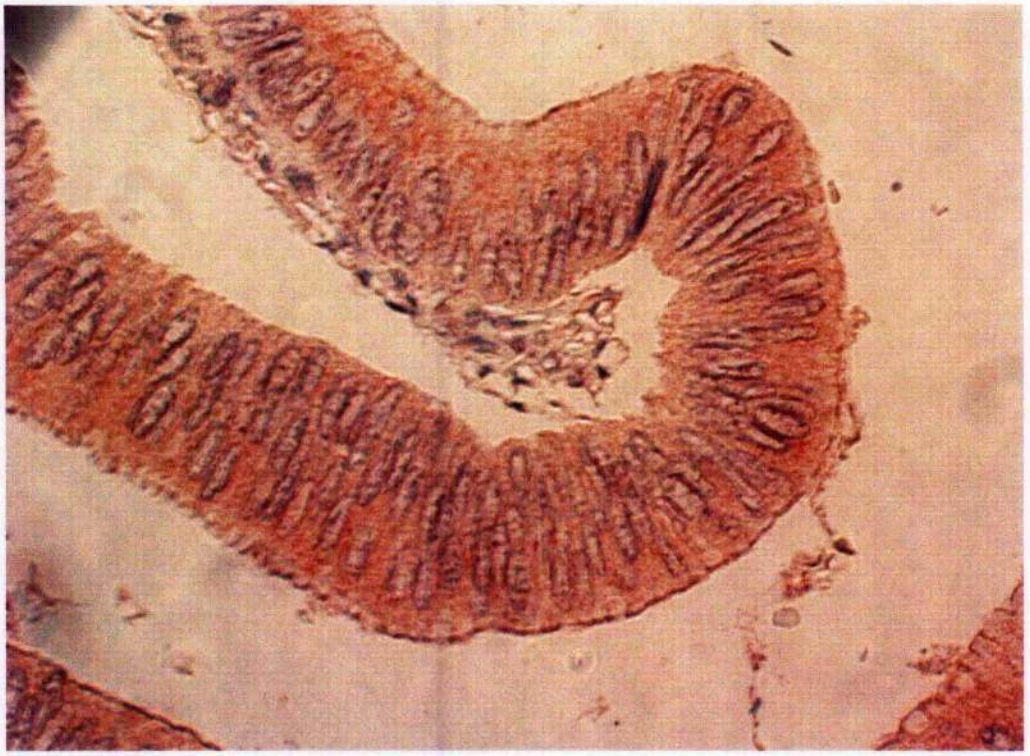


Figure 3.4 (c) Immunohistochemical staining of IL-6 antigen in tumour tissue (Goat IL-6 polyclonal antibody (EDTA)).

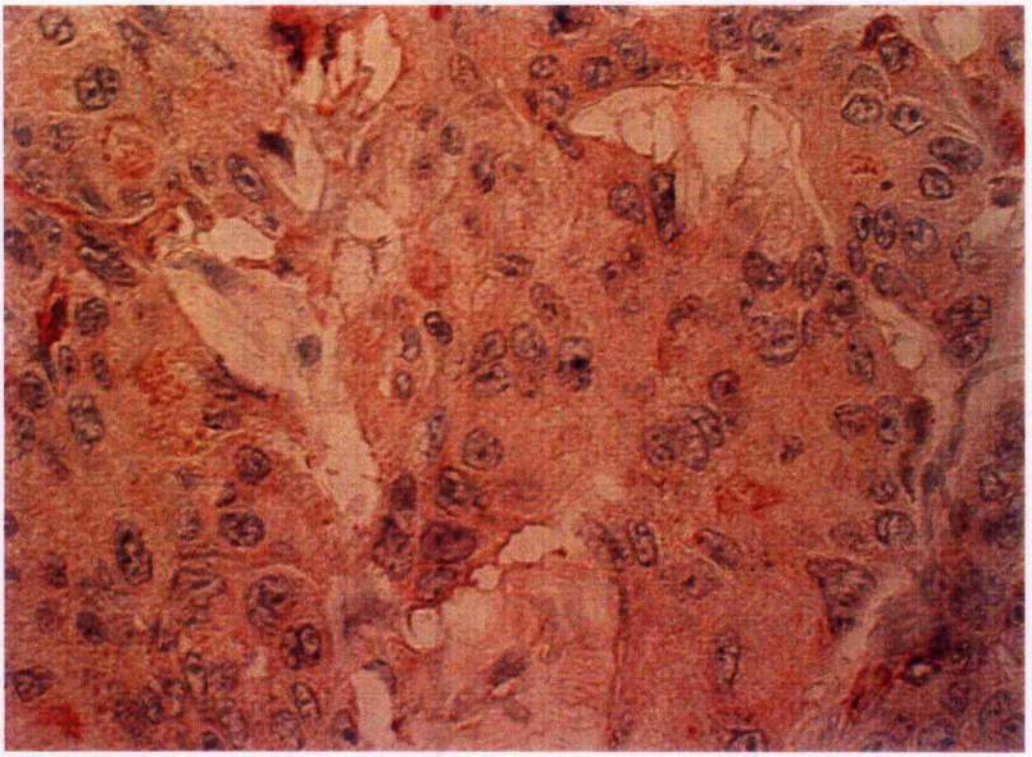
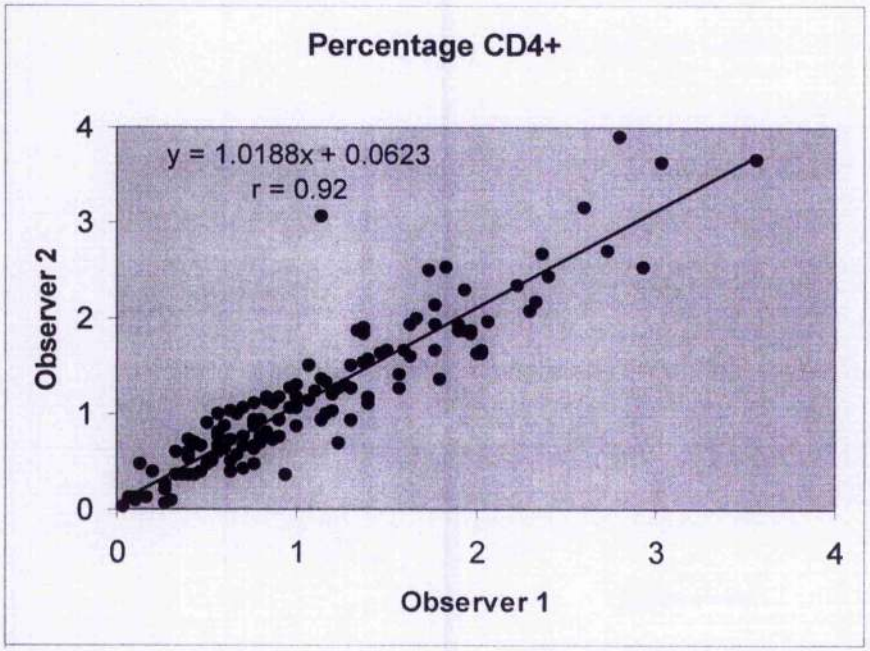


Figure 3.4 (d) Immunohistochemical staining of IL-6 antigen in tumour tissue (Goat IL-6 polyclonal antibody (citrate)).

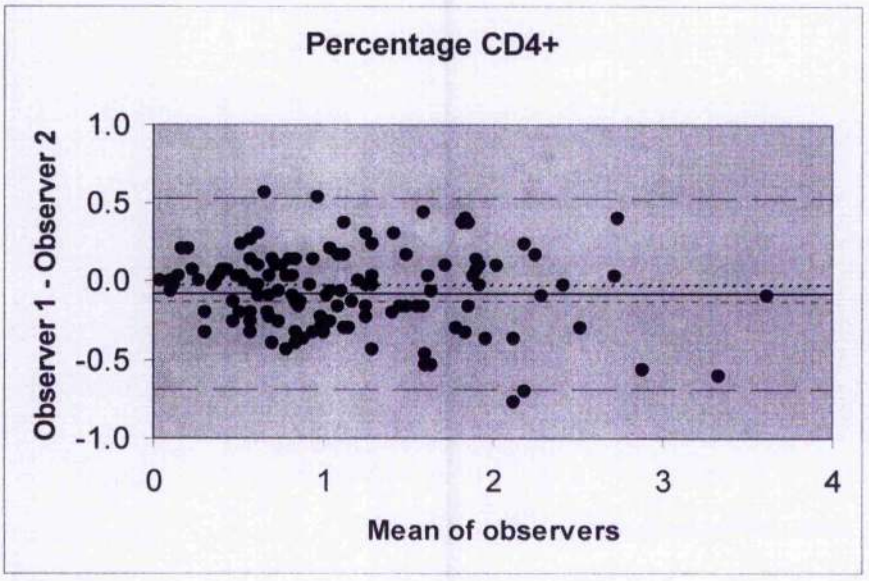
Table 3.1 **Inter-observer comparison of CD4+ and CD8+ lymphocyte volume density in 128 patients.**

	Observer 1 Mean	Observer 2 Mean	Difference Mean (95% CI)	P	R_i
CD4+ %	1.12	1.20	-0.08 (-0.14, -0.03)	0.003	0.92
CD8+ %	1.44	1.38	0.06 (-0.01, 0.14)	0.11	0.92

95% CI = 95% confidence interval, P = P-value, paired t-test, R_i = intra-class correlation coefficient.



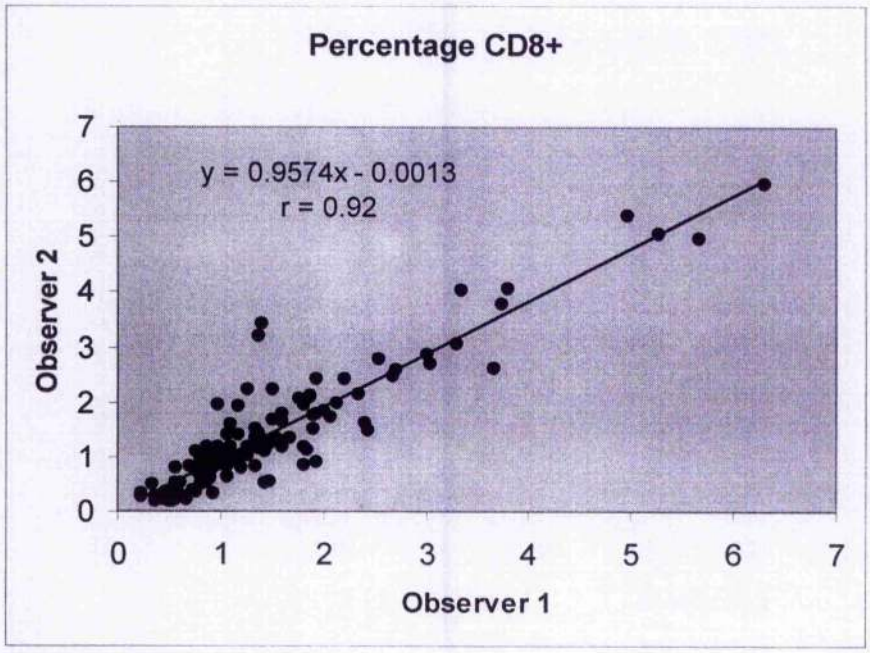
A



B

Figure 3.5

A. Relationship between independent measurements of CD4+ lymphocyte volume density by two observers. The equation of the least-squares linear regression line is shown together with the Pearson correlation coefficient. **B.** Bland-Altman plot of the difference between measurements of CD4+ lymphocyte volume density by two observers. The mean difference (solid line), its 95% confidence interval (fine dotted lines) and the limits of agreement within which 95% of individual differences would be expected to lie (coarse dotted lines) are also shown.



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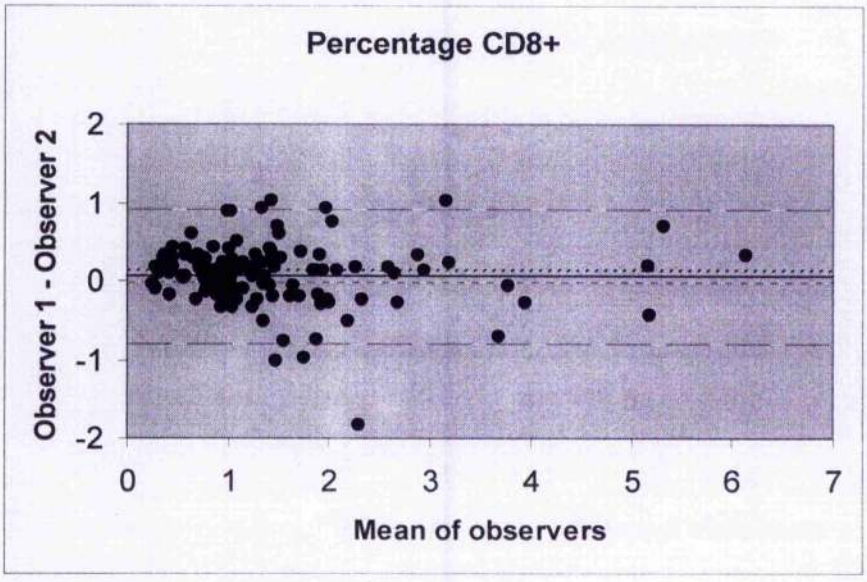
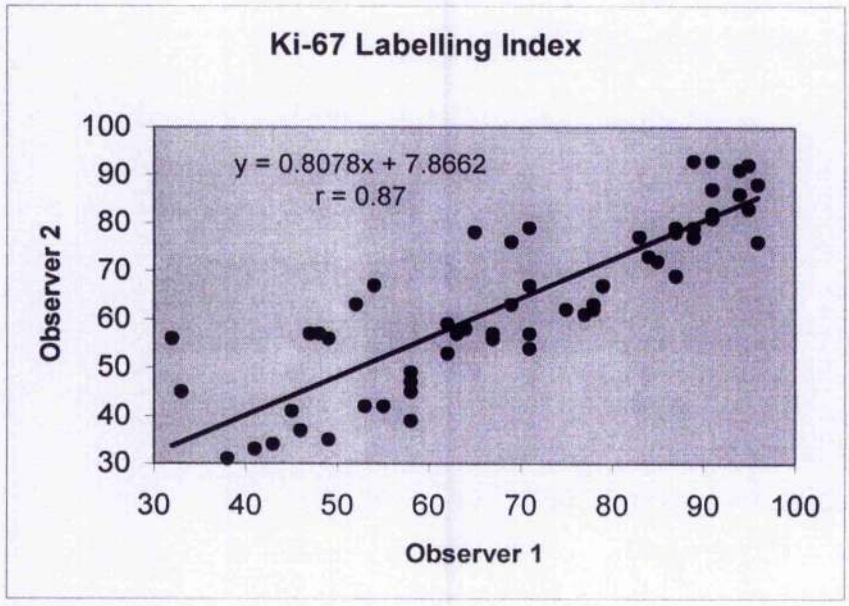


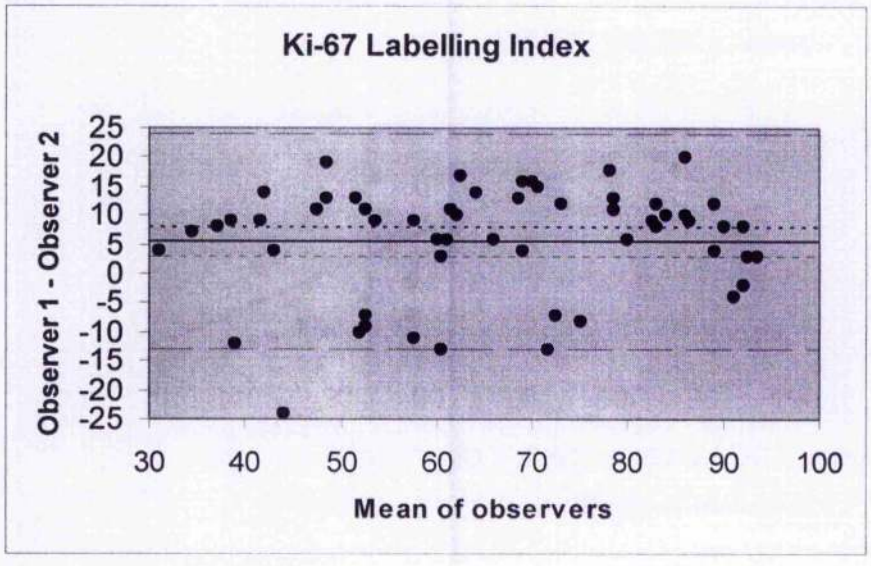
B

Figure 3.6

A. Relationship between independent measurements of CD8+ lymphocyte volume density by two observers. The equation of the least-squares linear regression line is shown together with the Pearson correlation coefficient. **B.** Bland-Altman plot of the difference between measurements of CD8+ lymphocyte volume density by two observers. The mean difference (solid line), its 95% confidence interval (fine dotted lines) and the limits of agreement within which 95% of individual differences would be expected to lie (coarse dotted lines) are also shown



A



B

Figure 3.7

A. Relationship between independent measurements of Ki-67 labelling index by two observers. The equation of the least-squares linear regression line is shown together with the Pearson correlation coefficient. **B.** Bland-Altman plot of the difference between measurements of Ki-67 labelling index by two observers. The mean difference (solid line), its 95% confidence interval (fine dotted lines) and the limits of agreement within which 95% of individual differences would be expected to lie (coarse dotted lines) are also shown.

CHAPTER FOUR

4.0 Evaluation of a simple prognostic score based on pathological stage and the systemic inflammatory response for patients with primary operable colorectal cancer

4.1 Introduction

Colorectal cancer is the second commonest cause of death from cancer in the United Kingdom. Each year in the UK, there are approximately 27,000 new cases and approximately 18,000 deaths attributable to the disease. Overall survival is poor, even in those who undergo potentially curative resection approximately 50% die within five years (McArdle and Hole, 2002).

The ideal prognostic score for patients undergoing potentially curative resection of a primary colorectal cancer should clearly distinguish those who will eventually succumb to the disease from those who are cured. Whilst Dukes' stage has been widely used, it fails to provide clear separation between these groups. Alternative factors which would provide additional information to that of Dukes' staging are therefore required.

Tumour progression is a complex process, which depends not only the intrinsic properties of the tumour, but also the tumour environment (Balkwill and Mantovani, 2001). One aspect of the host response, the non-specific systemic inflammatory response, is recognised to be an important factor in the progressive nutritional and functional decline of cancer patients (Kotler, 2000). Furthermore, there is accumulating evidence that the presence of a systemic inflammatory response (as evidenced by C-reactive protein) has prognostic significance in patients with colorectal cancer (McMillan et al., 1995; Nozoe et al., 1998; Nielsen et al., 2000; Wigmore et al., 2001;

Chung and Chang, 2003). However, some of these studies have questioned whether C-reactive protein has prognostic value independent of conventional pathological criteria including Dukes stage (Wigmore et al., 2001; Chung and Chang, 2003). Moreover, since in the above studies C-reactive protein was a significant prognostic factor, independent of Dukes stage, it may be that Dukes stage and C-reactive protein could be used to construct a new prognostic score.

The aim of the present study was to assess the value of combining Dukes stage and C-reactive protein to form a new prognostic score in patients undergoing apparently curative resection for colorectal cancer.

4.2 Patients and methods

Patients with Dukes B and C colorectal cancer, who, on the basis of laparotomy findings and pre-operative computed tomography, underwent curative resection between January 1997 and September 2001 in a single surgical unit at Glasgow Royal Infirmary, were included in the study. The tumours were staged using conventional Dukes' classification (Dukes and Bussey, 1958). Pre-operatively a blood sample was taken for the routine measurement of C-reactive protein. At this time no patient showed clinical evidence of tumour recurrence, infection, or other inflammatory conditions. Based on previous work a C-reactive protein concentration of greater than 10mg/l was considered to indicate the presence of a systemic inflammatory response (see section 3.2, chapter 3).

4.3 Statistical analysis

Data are presented as median and range. Comparisons between groups of patients were carried out using contingency table analysis (X^2). Grouping of the variables age and C-reactive protein was carried out using standard thresholds as shown in Table 4.1 (McArdle et al., 2003; Forrest et al., 2003; O'Gorman et al, 2000).

Survival analysis was performed using the Cox proportional hazard model. Deaths up to 31st December 2004 have been included in the analysis. Multivariate survival analysis was performed using a stepwise backward procedure to derive a final model of the variables that had a significant independent relationship with survival. To remove a variable from the model, the corresponding p-value had to be greater than 0.10. The survival curves in Figure 4.1 and survival rates in Table 4.2 were calculated using the

Kaplan-Meier technique. Analysis was performed using SPSS software (SPSS Inc., Chicago, Illinois, U.S.A.).

4.4 Results

The baseline clinicopathological characteristics of the patients (n=147) who underwent potentially curative surgery for colorectal cancer are shown in Table 4.1. Approximately one third of patients were aged 75 or over. The majority had colonic tumours, were Dukes stage B and had moderately differentiated tumours. Fifty four (36%) patients had an elevated C-reactive protein concentration prior to surgery.

The minimum follow-up was 40 months; the median follow-up of the survivors was 71 months. During this period 59 patients died 40 patients of their cancer and 19 of intercurrent disease.

On univariate analysis, increased age ($p<0.001$), Dukes stage C ($p<0.0001$), elevated circulating C-reactive protein concentrations ($p<0.0001$) and venous invasion ($p<0.01$) were associated with poor cancer specific survival.

On multivariate analysis, including the above variables, age (Hazard Ratio 1.96, 95%CI 1.26-3.04, p=0.0026), Dukes stage (HR 4.09, 95%CI 2.04-8.24, p=0.0001) and C-reactive protein (HR 4.38, 95%CI 2.11-9.09, p=0.0001) were significantly associated with cancer specific survival.

Since the magnitude of hazard ratios of Dukes stage (1.41) and C-reactive protein (1.48) were similar, this indicates that either an elevated CRP or the presence of a systemic

inflammatory response had approximately the same relative risk as an increase in pathological stage from Dukes B to Dukes C, and that they could be simply combined to form a prognostic score. Such a cumulative prognostic score was therefore constructed by assigning one point for each of the following criteria: Dukes stage C and C-reactive protein >10mg/l.

The relationship between stage, C-reactive protein concentration, the cumulative prognostic score and mortality are shown in Table 4.2. The relationship between the cumulative prognostic score and cancer specific survival is shown in Figure 4.1. The cancer specific survival rates at 3 years for patients with a cumulative prognostic score of 0, 1, 2 were 100%, 78% and 34% respectively (HR 5.02, 95%CI 3.09-8.17, $p<0.0001$).

4.5 Discussion

The ideal prognostic index for patients undergoing potentially curative resection of the primary colorectal cancer should clearly distinguish those who will eventually succumb to the disease from those who are cured. Whilst Dukes' stage has been widely used, it fails to provide clear separation between these groups. Alternative techniques, which would provide additional information to that of Dukes' staging, are therefore required.

There is a complex relationship between the primary tumour and the host response, which determines the likelihood of tumour recurrence (Balkwill and Mantovani, 2001). Clearly, in defining optimal prognostic factors both these aspects should be taken into account.

Several studies have shown that elevated circulating C-reactive protein concentrations are associated with poor survival in patients with colorectal cancer (McMillan et al., 1995; Nozoe et al., 1998; Nielsen et al., 2000; Wigmore et al., 2001; Chung and Chang, 2003). However, the relationship between C-reactive protein concentrations and conventional clinicopathological criteria is not clear since some of the above studies have included patients with Dukes A tumours who were unlikely to progress and patients with Dukes D tumours who had already progressed (Wigmore et al., 2001; Chung and Chang, 2003). This is likely to have confounded the assessment of the prognostic value of an elevated circulating C-reactive protein concentration.

Clinicians have long recognised that some high-risk Dukes B patients behave more like patients with Dukes C tumours. Not only has this implication for the prediction of survival but may also have implications for treatment. For example, there is evidence

that adjuvant chemotherapy is of benefit, at least in Dukes C colorectal cancer (IMPACT investigators, 1995, O'Connell et al., 1997). A third of these patients however, will survive 5 years without treatment, and would therefore receive potentially toxic therapy without benefit. In contrast, many patients with Dukes B tumours, almost one third of whom will die of their disease within 5 years, may fail to receive chemotherapy (McArdle and Hole, 2002).

In the present study, both Dukes stage and C-reactive protein concentrations were independently associated with cancer specific survival. These results are consistent with those of Nielsen and co-workers (2000) who, in a cohort of almost 400 patients undergoing resection for Dukes B and C tumours, also demonstrated that C-reactive protein was a Dukes stage independent prognostic factor. The mechanism by which a systemic inflammatory response might influence cancer survival is not clear. However, it is known that as part of the systemic inflammatory response, there is a release of pro-inflammatory cytokines and growth factors which may promote tumour growth (Abramovitch et al, 1999) and compromise immune function (Coussens and Werb, 2002).

In the present study, when C-reactive protein concentrations were combined with Dukes stage to form a new prognostic score, the combined score improved the prediction of cancer specific survival. The addition of C-reactive protein differentiated between low and high risk Dukes B and low and high risk Dukes C patients. Cancer related mortality ranged from 0% in patients with Dukes B tumours and a normal C-reactive protein concentration to 66% in patients with Dukes C tumours and an elevated C-reactive protein concentration. Cancer related mortality in patients with Dukes B tumours and

an elevated C-reactive protein concentration was similar to that of patients with Dukes C tumours and a normal C-reactive protein concentration. Therefore, this cumulative prognostic score may be useful in identifying high risk Dukes B patients for adjuvant therapy.

It is generally recognised that tumour stage, as reflected for example in the Dukes classification, is one of the most powerful predictors of final outcome in colorectal cancer patients (Deans et al, 1992). The overall 5-year survival rate of patients with colorectal cancer is approximately 60%. However, this differs greatly for the different stages (Table 1.5). For stage A (approximately 9 % of patients) 5-year survival exceeds 90% and, conversely, for Dukes D (approximately 15% of patients) this figure is between 2 and 5 % (McArdle and Hole, 2002). For stage B, however, which constitutes approximately 47 % of patients, 5-year survival is 70 % and for Dukes C (about 30 % of patients) this is approximately 45 %. The lack of prognostic precision in stages B and C hampers stratification of patients into subgroups which might benefit from additional adjuvant therapy. Pathologists have responded to this problem by introducing new parameters which might have independent prognostic significance. More detailed histological evaluation of the pattern of invasion into the bowel wall or into veins has been advocated.

For example, Hase and co-workers (1993) reported that irregular tumour cell budding at the invasive front of the tumour indicates more aggressive behaviour than a straight "pushing" tumour margin. Also, Yamazoe and co-workers (1992) investigated the depth of venous invasion as a predictor for liver metastasis. This parameter appeared to predict the occurrence of liver metastasis with high probability, especially in

combination with desmoplastic reaction, lymphocytic infiltration and depth of invasion. The extent of lymphocytic infiltration was also taken into account in the classification proposed by Shepherd and co-workers (1989). The latter classification has gained some popularity, but has not become generally accepted. An important limitation of classifications based upon detailed histopathological criteria to date is their lack of standardization and thus reproducibility. More recently, pathological TNM staging for colorectal cancer has been introduced and provides further discrimination in predicting cancer specific survival. However, in the time period of the present study TNM staging was not routinely carried out and therefore it remains to be determined whether the systemic inflammatory response, as evidenced by an elevated C-reactive protein concentration, will offer prognostic value in addition to TNM stage.

The results of the present study indicate that the combination of Dukes stage and the systemic inflammatory response, as evidenced by an elevated C-reactive protein concentration, which reflects both the contribution of the tumour and the host response, differentiates between low risk and high risk Dukes B and C tumours in patients undergoing potentially curative resection for colorectal cancer. This combined score of pathological stage and C-reactive protein has the advantage that of being simple to measure, routinely available and well standardised.

Table 4.1 Clinicopathological characteristics in patients with colorectal cancer: univariate survival analysis.

	Patients (n= 147)	Hazard ratio (95% CI)	p-value
Age group (<65/ 65-74/ ≥75)	46/ 44/ 57	2.13 (1.39-3.25)	0.0005
Sex (male/ female)	78/ 69	1.77 (0.94-3.31)	0.0749
Site (colon/ rectum)	105/ 42	1.39 (0.66-2.89)	0.3960
Dukes stage (B/ C)	91/ 56	4.34 (2.24-8.43)	<0.0001
C-reactive protein (≤10/ >10mg/l)	93/ 54	6.05 (3.05-12.01)	<0.0001
Tumour characteristics			
Diameter (mm)	49/ 49/ 49	0.90 (0.62-1.31)	0.5985
Tertile (range)	10-35		
Tertile (range)	35-50		
Tertile (range)	50-130		
Ulceration (no/ yes)	72/ 75	1.07 (0.58-2.00)	0.8212
Differentiation (well/ moderate/ poor)	18/ 116/ 13	1.45 (0.72-2.92)	0.2925
Lymphatic invasion (negative/ positive)	124/ 22	1.29 (0.57-2.91)	0.5469
Venous invasion (negative/ positive)	118/ 28	2.79 (1.46-5.35)	0.0020
Adjuvant therapy (no/ yes)	116/ 31	1.09 (0.52-2.30)	0.8108

Table 4.2 Prognostic score following curative resection for colorectal cancer.

Dukes stage		C-reactive protein		Cumulative	Patients	3 yr survival rate
Score		(mg/l)	Score	Score	(n)	(%)
B	0	≤10	0	0	62	100
		>10	1	1	29	75
C	1	≤10	0	1	31	81
		>10	1	2	25	34

Cumulative score obtained by adding scores for Dukes stage and C-reactive protein.

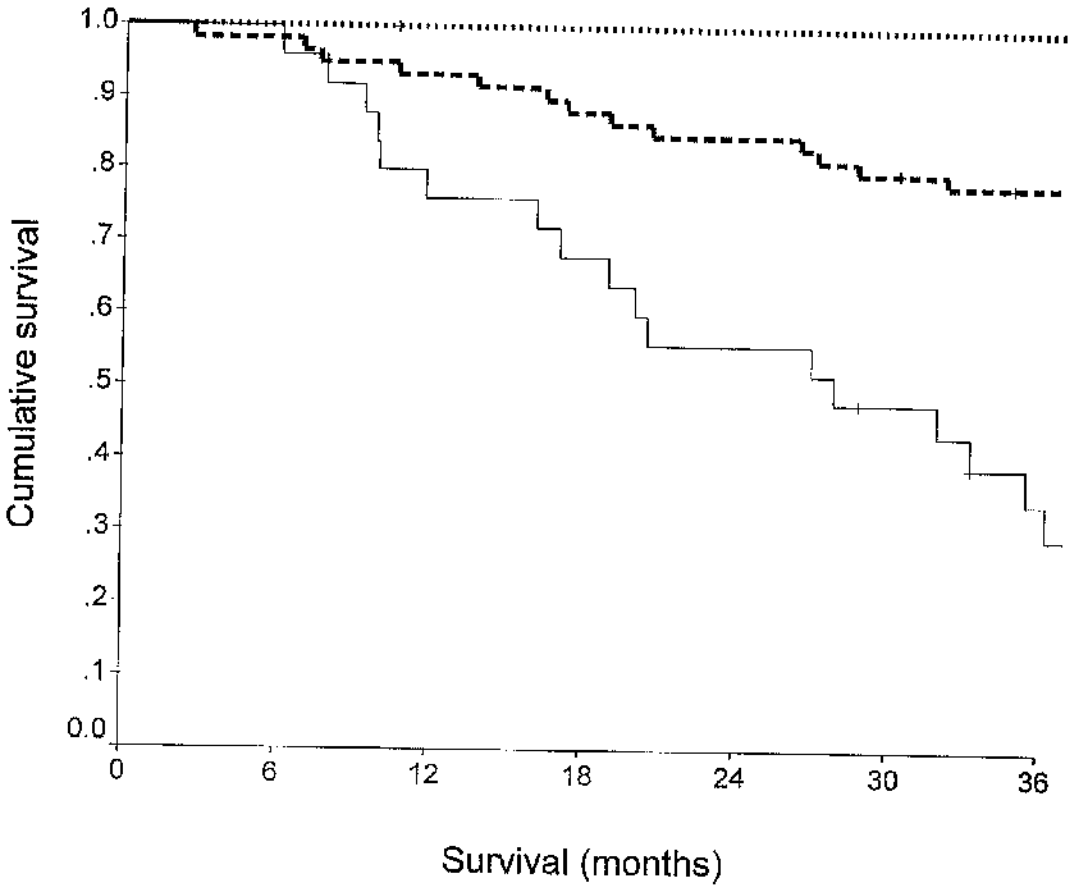


Figure 4.1 The relationship between the cumulative prognostic score (0 ..... , – – 1, —— 2) and cancer specific survival following potentially curative surgery for colorectal cancer.

CHAPTER FIVE

5.0 An investigation of the inter-relationships between the systemic inflammatory response, tumour lymphocytic infiltration, tumour proliferative activity and cancer survival in patients with primary operable colorectal cancer

5.1 Introduction

It has long been recognised that disease progression in cancer patients is not solely determined by the characteristics of the tumour but also by the host response. Indeed, there is increasing evidence that both local and systemic inflammatory responses play an important role in the progression of a variety of common solid tumours (O'Byrne and Dalgleish, 2001; Vakkila and Lotze, 2004; Pages et al., 2005).

In the previous chapter, we showed that the systemic inflammatory response, as evidenced by elevated circulating concentrations of C-reactive protein, was associated with poorer cancer specific survival, independent of Dukes stage. Circulating concentrations C-reactive protein appear to be primarily determined by concentrations of interleukin-6 (Gabay and Kushner, 1999). It is of interest therefore, that circulating IL-6 concentrations in patients with colorectal cancer are related to its production by the tumour (Komoda H et al., 1998; Piancatelli et al., 1999; Kinoshita et al., 1999). However, it remains unclear whether this interleukin-6 originates from the tumour cells themselves or from the inflammatory infiltrate. Nevertheless, there is some evidence that IL-6 acts as a proliferative factor in patients with colorectal cancer (Kinoshita et al., 1999).

From immunological point of view, the types of immune effectors that can reliably be considered as a sign of a systemic anti-tumour immune response are cytotoxic T

lymphocytes (CTLs), classically CD8+ T-lymphocytes. T-lymphocytes are the main effector arm of acquired and antigen-specific cellular immune responses, and play a central role both in the control of viral infections and in immunity against cancer (Melief et al, 1991; Riddell et al, 1995). In patients with colorectal cancer, there is good evidence that, on simple staining of tumour sections, the presence of a pronounced lymphocytic infiltration within the tumour is associated with improved survival (Jass et al., 1987; Ropponen et al., 1997; Nielsen et al., 1999). More recently, the ability to identify lymphocyte subsets reliably by immunohistochemistry has led to renewed interest in the relationship between the tumour inflammatory infiltrate and outcome. Indeed, increased infiltration of the tumour by CD8+ (Naito et al., 1998; Prall et al., 2004; Chiba et al., 2004) and CD4+ T-lymphocytes (Ali et al., 2004) has been shown to be associated with increased survival in patients with colorectal cancer.

In the studies to date, patients who were unlikely to progress (Dukes A) and patients who had already progressed (Dukes D) were included in the analysis. Therefore, the value of total and subset lymphocytic counts in patients with Dukes B and C tumour, in whom it is difficult to predict disease progression, remains uncertain.

One factor which may determine the pattern and extent of the tumour T-lymphocytic infiltration is the proliferative activity of the tumour. It has long been recognised that a high tumour proliferation rate is associated with aggressive malignant disease (Rosenwald, 2004). Indeed, it has been shown that increased expression of the protein Ki-67, a high molecular weight nuclear protein associated with cell division, is associated with an increased proliferation rate and poorer survival in a variety of common solid tumours including breast and lung cancer (Brown and Gatter, 2002).

However, the relationship between the expression of Ki-67 in the tumour and survival in colorectal cancer is less clear.

For example, in a recent review of those studies which examined the relationship between tumour expression of Ki-67 and outcome in colorectal cancer, only two out of 12 reported a significant association with survival (Brown and Gatter, 2002). However, many of these studies were based on small numbers, some included both primary operable and advanced disease and most used semi-quantitative techniques for the measurement of Ki-67.

To date, the inter-relationships between the systemic inflammatory response, tumour lymphocytic infiltration, tumour proliferative activity and cancer survival in patients with primary operable colorectal cancer do not appear to have been examined. Therefore, the aim of the present study was to examine the relationship between systemic inflammatory response, tumour lymphocytic infiltration, tumour proliferative activity and cancer specific survival in patients undergoing potentially curative resection for colorectal cancer

5.2 Patients & Methods.

Patients with Dukes B and C colorectal cancer, who, on the basis of laparotomy findings and pre-operative computed tomography, underwent curative resection between January 1997 and September 2001 in a single surgical unit at Glasgow Royal Infirmary were included in the study. The tumours were staged using conventional Dukes' classification (Dukes and Bussey, 1958). Pre-operatively a blood sample was taken for the routine measurement of C-reactive protein. At this time no patient showed clinical evidence of tumour recurrence, infection, or other inflammatory conditions. Based on previous work a C-reactive protein concentration of greater than 10mg/l was considered to indicate the presence of a systemic inflammatory response. Patients who had non-elective surgery or pre-operative radiotherapy or died within 30 days of surgery or showed clinical evidence of infection or other inflammatory conditions were excluded from the study (see section 3.1, Chapter 3).

Blocks from the primary tumour were fixed in 10% buffered formalin and embedded in paraffin wax. One representative block of tumour was selected for each patient. Serial sections (4 mm) were cut and mounted on slides coated with aminopropyltriethoxysilane.

CD4+ and CD+ T-lymphocytes (Figure 3.1& 3.2)

Sections were immunostained using the peroxidase-based Envision (Dako, Cambridgeshire, UK) technique as previously described (Section 3.3, Chapter 3). The primary antibody for CD4 was mouse monoclonal (Vector, Peterborough, UK) and that for CD8 was mouse monoclonal (Dako, Cambridgeshire, UK).

Ki-67 (Figure 3.3):

Sections were immunostained using the ChemMate Dako Envision method (Dako, Cambridgeshire, UK). The technique as previously described (Section 3.5, Chapter 3). The primary antibody for Ki-67 was mouse monoclonal antibody (Dako).

Morphometry

Quantitative analysis of the lymphoid infiltrate was performed using point counting (Anderson and Dunnill, 1965) with a random sampling technique (Section 3.4, Chapter 3). The Ki-67 labelling indexes were assessed using same method as previously described (Section 3.6, Chapter 3).

5.3 Statistical analysis

For the purpose of analysis, T-lymphocyte subsets and Ki-67 labelling index were grouped by tertiles. Relationships between T-lymphocyte counts and other variables were analysed using the Mantel-Haenszel chi squared test for trend and Spearman rank correlation analysis as appropriate. Survival analysis was performed using the Cox proportional hazard model.

The degree of agreement between observers in the measurement of CD4+ and CD8+ lymphocyte volume density was expressed by the intra-class correlation coefficient (Bartko and Carpenter 1976). A high value for the conventional correlation coefficient, which is a measure of linear association, does not necessarily imply close agreement: for example, there may be a consistent additive or multiplicative bias between the observers. By contrast, the intra-class correlation coefficient (R_i) is a measure of true agreement. The conventional interpretation of R_i is as follows: R_i<0.40, poor agreement; R_i=0.40-0.59, fair agreement; R_i=0.60-0.74, good agreement; $R_i \geq 0.75$, excellent agreement. . In the present study the intra-class coefficient for measurement of CD4+, CD8+ and Ki-67 labelling index were greater than 0.84 (see sections 3.4 & 3.6, chapter 3)

Bias between observers was assessed using the paired t-test, a p-value of 0.05 or less being considered statistically significant. Bland-Altman plots of the inter-observer difference against the mean of the two observers' measurements were also constructed (see sections 3.4 & 3.6, chapter 3).

Survival (cancer-specific) analysis was performed using the Cox proportional hazard model. Deaths up to 31st December 2004 have been included in the analysis. Multivariate survival analysis was performed using a stepwise backward procedure to derive a final model of the variables that had a significant independent relationship with survival. To remove a variable from the model, the corresponding P-value had to be greater than 0.10. Analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA).

5.4 Results

The baseline clinicopathological characteristics of the patients (n=147) who underwent potentially curative surgery for colorectal cancer are shown in Table 5.1. Approximately one third of patients were aged 75 or over. The majority had colonic tumours, were Dukes stage B and 54 (36%) patients had an elevated C-reactive protein concentration prior to surgery. The majority of patients had haemoglobin, white cell, lymphocyte and platelet counts were in the normal range.

The minimum follow-up was 40 months; the median follow-up of the survivors was 71 months. During this period 59 patients died 40 patients of their cancer and 19 of intercurrent disease. On univariate analysis, increased age ($p<0.001$), sex ($p<0.10$), Dukes' stage ($p<0.0001$), elevated circulating C-reactive protein concentrations ($p<0.0001$), venous invasion ($p<0.01$), increased Ki-67 labelling index ($p<0.01$), decreased percentage tumour volume of CD+4 ($p<0.05$) and CD8+ ($p<0.10$) T-lymphocytes were associated with poor cancer-specific survival.

Patients were grouped according to absence or presence of a systemic inflammatory response (C-reactive protein ≤10/ >10mg/l respectively) are shown in Table 5.2. The groups were similar in terms of sex, tumour site, stage, haemoglobin, lymphocyte, platelet counts and tumour characteristics. Those patients with evidence of a systemic inflammatory response were older ($p<0.01$), had higher white cell counts ($p<0.10$), larger tumour diameter ($p<0.001$), more ulcerative tumours ($p<0.10$), more moderate and poor tumour differentiation ($p<0.10$), a higher Ki-67 labelling index ($p<0.05$), a lower tumour CD4+ lymphocytic infiltration ($p<0.001$). There was a significant correlation between the percentage tumour CD4+ and CD8+ T-lymphocytes ($r_s= 0.44$,

p<0.001). There was an inverse relationship between percentage tumour CD4+ T-lymphocytes and C-reactive protein (r_s= -0.26, p=0.003, Figure 5.1). There was no significant relationship between percentage tumour CD8+ T-lymphocyte and C-reactive protein (r_s= -0.09, p=0.273, Figure 5.2).

Patients were grouped according to tertiles of the percentage tumour volume of CD4+ T-lymphocytes are shown in Table 5.3. The groups were similar in terms tumour site, stage, white cell, lymphocyte and platelet counts and tumour characteristics. Those patients with a high tumour CD4+ lymphocytic infiltration were older (p<0.10), more likely to be male (p<0.05), had a normal C-reactive protein (p<0.001) and haemoglobin (p<0.05) concentrations, a smaller tumour diameter (p<0.05) and a higher tumour CD8+ lymphocytic infiltration (p<0.001).

Patients were grouped according to tertiles of the Ki-67 labelling index are shown in Table 5.4. The groups were similar in terms age, tumour site, stage, haemoglobin, white cell, lymphocyte and platelet counts and tumour characteristics. Those patients with a high tumour Ki-67 labelling index, more likely to be female (p<0.05), had an elevated C-reactive protein (p<0.05, Figure 5.3) and a larger tumour diameter (p<0.01).

On multivariate analysis of the significant covariates (Table 5.5), only age (p<0.01), sex (p<0.05), stage (p<0.001), C-reactive protein (p<0.001) and tumour CD8+ lymphocytic infiltration (p<0.01) retained independent significance.

5.5 Discussion

In the present study, confined to patients with Dukes B and C colorectal cancer undergoing potentially curative resection, a number of factors were associated with poor cancer specific survival. In particular, a poor tumour CD4+ and CD8+ T-lymphocyte infiltrate and increased Ki-67 labelling index were associated with poorer cancer specific survival. Poor tumour CD4+ T-lymphocyte infiltrate was associated with an elevated circulating C-reactive protein and haemoglobin concentration but not tumour characteristics. Similarly, increased Ki-67 labelling index was associated with an elevated circulating C-reactive protein, tumour diameter but not other tumour characteristics. An elevated C-reactive protein, in addition to being associated with poor tumour CD4+ T-lymphocyte infiltrate and increased Ki-67 labelling index was also associated with tumour diameter. Taken together these results would suggest that local and systemic inflammatory responses are linked to tumour proliferation in patients with colorectal cancer and these responses are in turn linked to cancer specific survival, independent of tumour stage.

In order to examine the relative importance of these factors we carried out multivariate survival analysis. This showed that both local (tumour CD8+ T-lymphocyte infiltrate) and systemic (C-reactive protein) inflammatory responses, but not tumour proliferation (Ki-67 labelling index), were significantly associated with cancer specific survival. The reasons that, compared with the inflammatory responses, tumour proliferation is a relatively poor predictor of survival possibly relates to the fact that tumour dissemination, rather than tumour proliferation, is the primary determinant of survival in patients with colorectal cancer.

With respect to the relationship between the Ki-67 labelling index and survival the results of the present study appear to be in contrast with those of previous studies. For example, Brown and Gatter (2002) in their recent review of those studies, only three out of 12 studies reported a significant association with survival (Palmqvist et al, 1999; Saleh et al, 1999; Kimura et al, 2000). Indeed, the result of above studies which did show a correlation were also contradictory; for example, Palmqvist and co-workers (1999) concluded that colorectal carcinomas with low Ki-67 protein expression at the invasive margin had a poor prognosis, whilst Saleh and co-workers (1999) and Kimura and co-workers (2000) showed that a high Ki-67 protein expression at the site of invasive margin had a worse prognosis. These apparently contradictory findings may be the result of the marked heterogeneity of Ki-67 protein expression in colorectal carcinomas (Ofner et al, 1996). However, many of these studies were based on small numbers; some included both primary operable and advanced disease and most used semi-quantitative techniques for the measurement of Ki-67. Indeed, some of the largest studies included both patients with primary operable and advanced disease (Jansson and Sun, 1997; Buglioni et al., 1999).

In the present study, a minimum of 1000 tumour cells in randomly selected fields were counted in order to assess the percentage of Ki-67 positively staining nuclei. In contrast, most of the largest studies to date have used semi-quantitative methods to assess Ki-67 expression (Jansson and Sun, 1997; Bhatavbekar et al., 2001; Allegra et al., 2002). Therefore, it is likely that the method used in the present study offers a more accurate assessment of Ki-67 expression. Recently, Valera and co-workers (2005) reported that, using a double immunohistochemical staining procedure and a quantitative technique in a cohort of 106 patients who underwent curative resection for

colorectal cancer, an increased Ki-67 proliferation index was associated with poorer survival and it maintained significance as an independent predictor of long-term outcome in a multivariate analysis that included other prognostic factors. This study also showed a significant correlation between proliferative index and several pathological characteristics of colorectal carcinomas, such as tumour differentiation, metastatic disease and local invasiveness in keeping with earlier studies (Porschen et al, 1991; Kubota et al, 1992; Diebold et al, 1994; Kyzer et al,1997).

These results would confirm that although Ki-67 expression reflects the proliferation rate of the tumour, it does not reflect the ability of the tumour to disseminate. These results are consistent with the concept that tumour proliferation alone does not determine outcome in the cancer patient (Balkwill and Mantovani, 2001; Vakkila et al, 2004).

In the present study there was a direct relationship between the Ki-67 labelling index and C-reactive protein concentrations. These results are consistent with the concept that C-reactive protein production is primarily determined by interleukin-6 which in turn stimulates tumour cell proliferation in colorectal cancer (Kinoshita et al, 1999). In the present study, we were unable to reliably identify regions of Il-6 expression in the colorectal tumours due to deep background staining which precluded accurate scoring of IL-6 positive cells in the tumour tissue. This was despite using different methods of antigen retrieval and staining and the use of negative and positive controls. The reasons for the poor quality of immuno-staining are unclear. However, on inspection of the equivalent Il-6 staining by Kinoshita and co-workers (1999) it would appear likely that non-specific binding by the primary antibody was a problem.

Like most epithelial solid tumours, colorectal carcinoma has long been considered poorly immunogenic and substantially refractory to immunotherapy. This opinion was based on indirect data from: (a) epidemiological studies on lack of spontaneous regression of cancer; (b) in vitro studies on tumour infiltrating lymphocytes (TIL); (c) a first generation of clinical trials of immunotherapy in colorectal cancer patients; (d) in colorectal carcinoma spontaneous regression is only exceptionally observed, and does not appear to be associated with an immune response (Papac et al, 1996; Francis et al, 1997).

During the last decade, however, continuous progress in the molecular characterisation of T cell-defined tumour associated antigens (TAA) and in methods allowing detection of antigen-specific T cell responses have slowly modified the scientific community's perspective. One classical way to evaluate if anti-tumour immune responses are taking place in vivo in cancer patients, and can influence the natural history of the disease, is to perform histopathological evaluation of surgically resected tumour lesions looking for immune effectors within tumour tissue, and trying to correlate their presence with patient prognosis. To avoid confusion, pathological evaluation of intra-tumour inflammatory infiltrates should take into careful consideration some important parameters, such as cellular composition and anatomical localisation.

A key to understand the complex local inflammatory response in colorectal cancer could reside in accurate and qualitative classification of inflammatory infiltrates, taking into careful consideration both the type of recruited immune effectors and their anatomical localization within the tumour tissues (intra-epithelial vs. stromal or peri-tumoural). The results of the present study are consistent with those of Nielsen and co-workers

(1999) who showed, on simple staining of tumour sections, that a poor inflammatory infiltrate including lymphocytes was associated with poorer survival. Also, they appear to be consistent with previous work by Naito and co-workers (1998) who showed that, in 131 tumours, tumour CD8+ T-lymphocyte infiltrate had prognostic value in patients with colorectal cancer. They divided CD8+ T-lymphocytes into three groups: (a) peri-tumoural, when distributed along the invasive margin of the tumour; (b) stromal, when infiltrating the tumour stroma; and (c) intra-epithelial, when infiltrating within cancer cell nests and taking direct contact with tumour cells. They reported that the presence of peri-tumoural and stromal CD8+ T lymphocytes was not associated with improved prognosis in multivariate analysis, while the presence of intra-epithelial CD8+ T lymphocytes was a strong predictor of better survival independently of Duke's stage. In the present study tumour T-lymphocyte subset density was assessed using a point counting technique. This approach provided an objective assessment of lymphocytic infiltration and circumvents the problem of variation in distribution of lymphocytes within an individual tumour. Furthermore, the study of Naito and colleagues (1998) included patients with Dukes A tumours who were unlikely to progress and patients with Dukes D tumours who had already progressed.

The relationship between tumour CD4+T-lymphocytic infiltration and cancer specific survival is the opposite of that previously reported for both renal and prostate cancer (Bromwich et al., 2003; McArdle et al., 2004a). The reasons for this are as yet unclear. However, given that tumour lymphocytic infiltration parallels that of other inflammatory cells (Nielsen et al., 1999; Lin and Pollard, 2004) and that an elevated C-reactive protein is associated with poor outcome in all three tumours (Blay et al., 1992; Lewenhaupt et al, 1990; McMillan et al, 2003) it appears likely that the source of interleukin-6, the primary stimulus to C-reactive protein, (Gabay and Kushner, 1999) is

different in different tumours. Indeed, McArdle and coworkers (2004b) have recently reported that the relationship between interleukin-6 and C-reactive protein was similar in benign prostatic hyperplasia and prostate cancer and that there was no relationship between interleukin-6 and PSA concentrations. This would suggest that interleukin-6 was coming from inflammatory cells. In colorectal cancer, however, it has been reported that interleukin-6 concentrations increase with tumour stage and correlate with CEA concentrations and this might, therefore suggest that interleukin-6 is coming from tumour cells (Kinoshita et al., 1999; Belluco et al., 2000; Miki et al., 2004). If this were to prove to be the case it would have important implications for the treatment of the systemic inflammatory response in patients with different cancers.

It was also of interest that C-reactive protein (a marker of the systemic inflammatory response) was also independently associated with cancer specific survival. Clearly, C-reactive protein can be measured with greater accuracy and precision than tumour T-lymphocytic infiltration. Nevertheless, it would appear from the present results that the systemic inflammatory response is also important in determining survival in these patients. The reasons for the importance of the systemic inflammatory response, independent of the local inflammatory response, are not clear. There are a number of possible explanations. Firstly, that C-reactive protein plays a more pivotal role in the tumour-host relationship. An elevated C-reactive protein concentration may identify those patients with a proangiogenic environment, since increased angiogenesis is associated with poor outcome in patients with colorectal cancer (Salmon et al, 2005) and circulating concentrations of vascular endothelial growth factor are directly associated with C-reactive protein (Xavier et al, 2006). Secondly, that C-reactive protein is recognised to be an activator of innate immunity and a modulator of adaptive

immunity (Du Clos and Mold, 2004) and its elevation is a precursor to progressive involuntary loss of weight and lean tissue which are key factors in determining cancer survival (McMillan et al., 1999, Kotler, 2000, Van Cutsem and Arends, 2005). Clearly, both local and systemic inflammatory mechanisms may be related and promote unrestrained tumour growth and the dissemination required for the greater malignant potential associated with these inflammatory response.

In summary, the results of the present study show that both an increased tumour Ki-67 labelling index and a low CD8+ T-lymphocyte count are independently associated with poorer outcome in patients undergoing curative resection for colorectal cancer. C-reactive protein, a mediator of the inate immune response, also had prognostic value independent of Dukes stage. Taken together these results may suggest that upregulation of the innate immune response and down regulation of cell mediated immunity is important in determining poorer survival in colorectal cancer

Table 5.1 The relationship between clinicopathological characteristics and cancer specific survival in patients undergoing potentially curative resection for colorectal cancer: Univariate survival analysis.

	Patients (n= 147)	Hazard ratio (95% CI)	p-value
Age group (<65/ 65-74/ ≥75)	46/ 44/ 57	2.13 (1.39-3.25)	0.0005
Sex (male/ female)	78/ 69	1.77 (0.94-3.31)	0.0749
Site (colon/ rectum)	105/ 42	1.39 (0.66-2.89)	0.3960
Dukes stage (B/ C)	91/ 56	4.34 (2.24-8.43)	0.0001
C-reactive protein (≤10/ >10mg/l)	93/ 54	6.05 (3.05-12.01)	0.0001
Haemoglobin (≥12/ <12g/dl)	69/ 78	1.16 (0.62-2.16)	0.6438
White cell count (<8.5/ 8.5-11.0/ >11.0 x10^9)	93/ 30/ 24	0.80 (0.52-1.25)	0.3310
Lymphocyte percentage (20-40/ 12-19.9/ 0-11.9)	73/ 35/ 39	1.06 (0.74-1.53)	0.7466
Platelets (<400/ >400 x10^9)	126/ 21	0.83 (0.33-2.13)	0.7050
Tumour characteristics			
Diameter (tertiles 1, 2, 3)	40 (10-130)*	0.90 (0.62-1.31)	0.5985
Ulceration (no/ yes)	72/ 75	1.07 (0.58-2.00)	0.8212
Differentiation (well/ moderate/ poor)	18/ 116/ 13	1.45 (0.72-2.92)	0.2925
Lymphatic invasion (negative/ positive)	124/ 22	1.29 (0.57-2.91)	0.5469
Venous invasion (negative/ positive)	118/ 28	2.79 (1.46-5.35)	0.0020
Ki-67 labelling index (tertiles 1, 2, 3)	0.72 (0.32-0.96)*	1.79 (1.20-2.69)	0.0047
CD4+ T-lymphocytes (tertiles 1, 2, 3)	0.90 (0.03-3.57)*	0.64 (0.43-0.95)	0.0272
CD8+ T-lymphocytes (tertiles 1, 2, 3)	1.13 (0.23-6.30)*	0.68 (0.46-1.01)	0.0541
CD4+plus CD8+ T-lymphocytes (tertiles 1, 2, 3)	2.17 (0.50-8.27)*	0.67 (0.45-0.99)	0.0426
Adjuvant therapy (no/ yes)	116/ 31	1.09 (0.52-2.30)	0.8108
Alive/ dead	88/ 59		
Cancer specific/ intercurrent disease	40/ 19		

* Median (range)

Table 5.2 The relationship between an elevated C-reactive protein concentration and clinicopathological characteristics in patients undergoing potentially curative resection for colorectal cancer.

	C-reactive protein ≤10mg/l (n= 93)	C-reactive protein >10mg/l (n= 54)	p-value
Age group (<65/ 65-74/ ≥75)	33/34/26	13/10/31	0.004
Sex (male/ female)	52/41	26/28	0.365
Site (colon/ rectum)	30/63	12/42	0.196
Dukes stage (B/ C)	62/31	29/25	0.120
Haemoglobin (≥12/ <12g/dl)	46/47	23/31	0.423
White cell count (<8.5/ 8.5-11.0/ >11.0 $x10^9$)	65/14/14	28/16/10	0.099
Lymphocyte percentage (20-40/ 12-19.9/ 0-11.9)	51/19/23	22/16/16	0.189
Platelets (<400/ >400 $x10^9$)	81/12	45/9	0.531
Tumour characteristics			
Diameter (tertiles 1, 2, 3)	41/29/23	8/20/26	0.0001
Ulceration (no/ yes)	51/42	21/33	0.063
Differentiation (well/ moderate/ poor)	14/73/6	4/43/7	0.072
Lymphatic invasion (negative/ positive)	80/12	44/10	0.374
Venous invasion (negative/ positive)	77/15	41/13	0.251
Ki-67 labelling index (tertiles 1, 2, 3)	37/31/25	12/18/24	0.012
CD4+ T-lymphocytes (tertiles 1, 2, 3)	22/32/39	27/17/10	0.0001
CD8+ T-lymphocytes (tertiles 1, 2, 3)	30/29/34	19/20/15	0.404
CD4+plus CD8+ T-lymphocytes (tertiles 1, 2, 3)	26/29/38	23/20/11	0.871
Adjuvant therapy (no/ yes)	73/20	43/20	0.871

Table 5.3 The relationship between CD+ T-lymphocyte tumour infiltration and clinicopathological characteristics in patients undergoing potentially curative resection for colorectal cancer.

	CD4+ (Tertile 1)	CD4+ (Tertile 2)	CD4+ (Tertile 3)	p-value
Age group (<65/ 65-74/ ≥75)	12/13/24	18/11/20	16/20/13	0.070
Sex (male/ female)	22/27	23/26	33/16	0.026
Site (colon/ rectum)	14/35	13/36	15/34	0.824
Dukes stage (B/ C)	30/19	33/16	28/21	0.678
C-reactive protein (≤10/ >10mg/l)	22/27	32/17	39/10	0.0001
Haemoglobin (≥12/ <12g/dl)	18/31	21/28	30/19	0.015
White cell count (<8.5/ 8.5-11.0/ >11.0 x10^9)	32/8/9	30/13/6	31/9/9	0.894
Lymphocyte percentage (20-40/ 12-19.9/ 0-11.9)	21/12/16	25/12/12	27/11/11	0.188
Platelets (<400/ >400 x10^9)	44/5	43/6	39/10	0.150
Tumour characteristics				
Diameter (tertiles 1, 2, 3)	12/19/18	15/14/20	22/16/11	0.036
Ulceration (no/ yes)	21/28	22/27	29/20	0.107
Differentiation (well/ moderate/ poor)	6/38/5	7/37/5	5/41/3	0.826
Lymphatic invasion (negative/ positive)	40/8	43/6	41/8	0.966
Venous invasion (negative/ positive)	38/10	42/7	38/11	0.835
Ki-67 labelling index (tertiles 1, 2, 3)	16/15/18	15/17/17	18/17/14	0.549
CD8+ T-lymphocytes (tertiles 1, 2, 3)	30/12/7	11/22/16	8/15/26	0.0001
Adjuvant therapy (no/ yes)	43/6	40/9	33/16	0.014

Table 5.4 The relationship between increasing Ki-67 labelling index and tumour characteristics in patients undergoing potentially curative resection for colorectal cancer.

	Ki-67 (Tertile 1)	Ki-67 (Tertile 2)	Ki-67 (Tertile 3)	p-value
Age group (<65/ 65-74/ ≥75)	18/14/17	14/20/15	14/10/25	0.147
Sex (male/ female)	26/23	35/14	17/32	0.069
Site (colon/ rectum)	14/35	13/36	15/34	0.824
Dukes stage (B/ C)	30/19	33/16	28/21	0.678
C-reactive protein (≤10/ >10mg/l)	37/12	31/18	25/24	0.012
Haemoglobin (≥12/ <12g/dl)	23/26	23/26	23/26	1.000
White cell count (<8.5/ 8.5-11.0/ >11.0 x10^9)	32/9/9	32/6/11	29/15/5	1.000
Lymphocyte percentage (20-40/ 12-19.9/ 0-11.9)	27/10/12	23/13/13	23/12/14	0.473
Platelets (<400/ >400 x10^9)	39/10	43/6	44/5	0.150
Tumour characteristics				
Diameter (tertiles 1, 2, 3)	25/16/8	11/15/23	13/18/18	0.007
Ulceration (no/ yes)	25/24	25/24	22/27	0.546
Differentiation (well/ moderate/ poor)	5/41/3	7/38/4	6/37/6	0.660
Lymphatic invasion (negative/ positive)	39/9	45/4	40/9	0.966
Venous invasion (negative/ positive)	38/10	41/8	39/10	0.961
CD4+ T-lymphocytes (tertiles 1, 2, 3)	16/15/18	15/17/17	18/17/17	0.459
CD8+ T-lymphocytes (tertiles 1, 2, 3)	17/17/15	16/16/17	16/16/17	0.711
CD4+plus CD8+ T-lymphocytes (tertiles 1, 2, 3)	16/16/17	18/13/18	15/20/14	0.805
Adjuvant therapy (no/ yes)	39/10	37/12	40/9	0.805

Table 5.5 The relationship between clinicopathological characteristics and cancer specific survival in patients undergoing potentially curative resection for colorectal cancer: Multivariate survival analysis.

	Patients (n= 147)	Hazard ratio (95% CI)	p-value
Age group (<65/ 65-74/ ≥75)	46/ 44/ 57	2.10 (1.34-3.29)	0.0013
Sex (male/ female)	78/ 69	2.13 (1.06-4.28)	0.0329
Dukes stage (B/ C)	91/ 56	4.28 (2.11-8.68)	0.0001
C-reactive protein (≤10/ >10mg/l)	93/ 54	4.39 (2.11-9.10)	0.0001
Tumour characteristics			
Venous invasion (negative/ positive)	118/ 28	1.97 (0.98-3.94)	0.0569
Ki-67 labelling index (tertiles 1, 2, 3)	0.72 (0.32-0.96)*	1.27 (0.84-1.92)	0.2628
CD4+ T-lymphocytes (tertiles 1, 2, 3)	0.90 (0.03-3.57)*	1.00 (0.63-1.89)	0.9902
CD8+ T-lymphocytes (tertiles 1, 2, 3)	1.13 (0.23-6.30)*	0.56 (0.36-0.85)	0.0075

* Median (range)

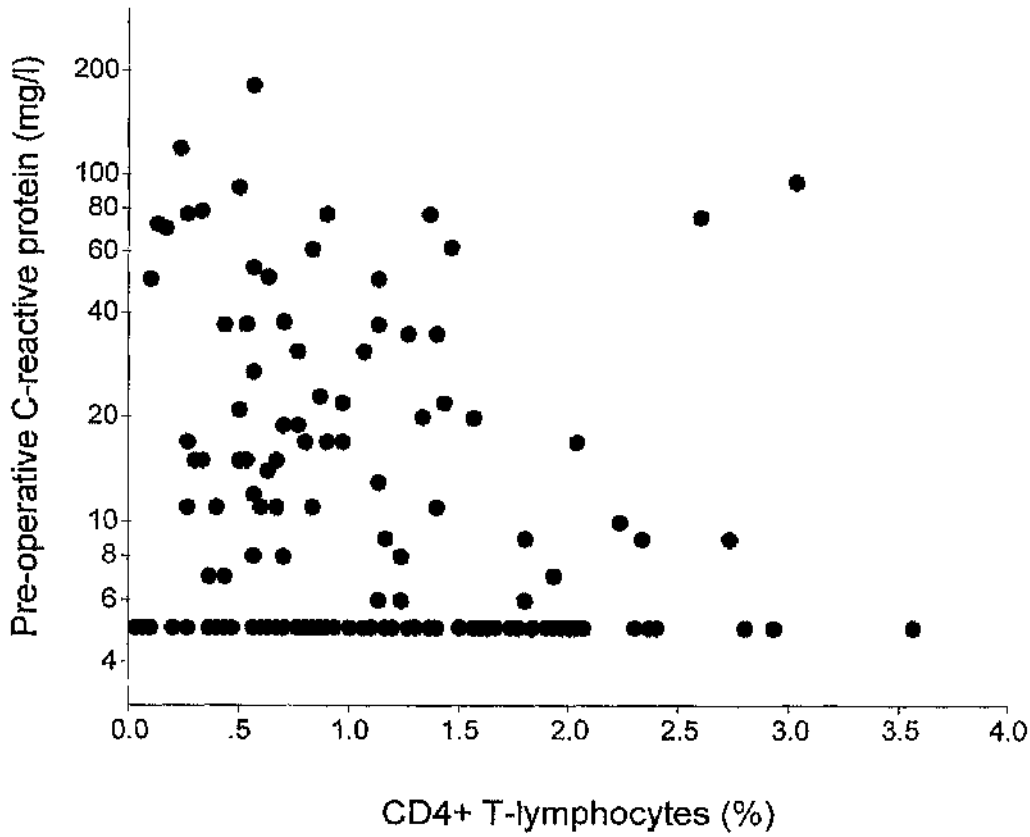


Figure 5.1 **Relationship between percentage tumour CD4+ T-lymphocyte infiltration and preoperative C-reactive protein in patients undergoing potentially curative resection for colorectal cancer.**

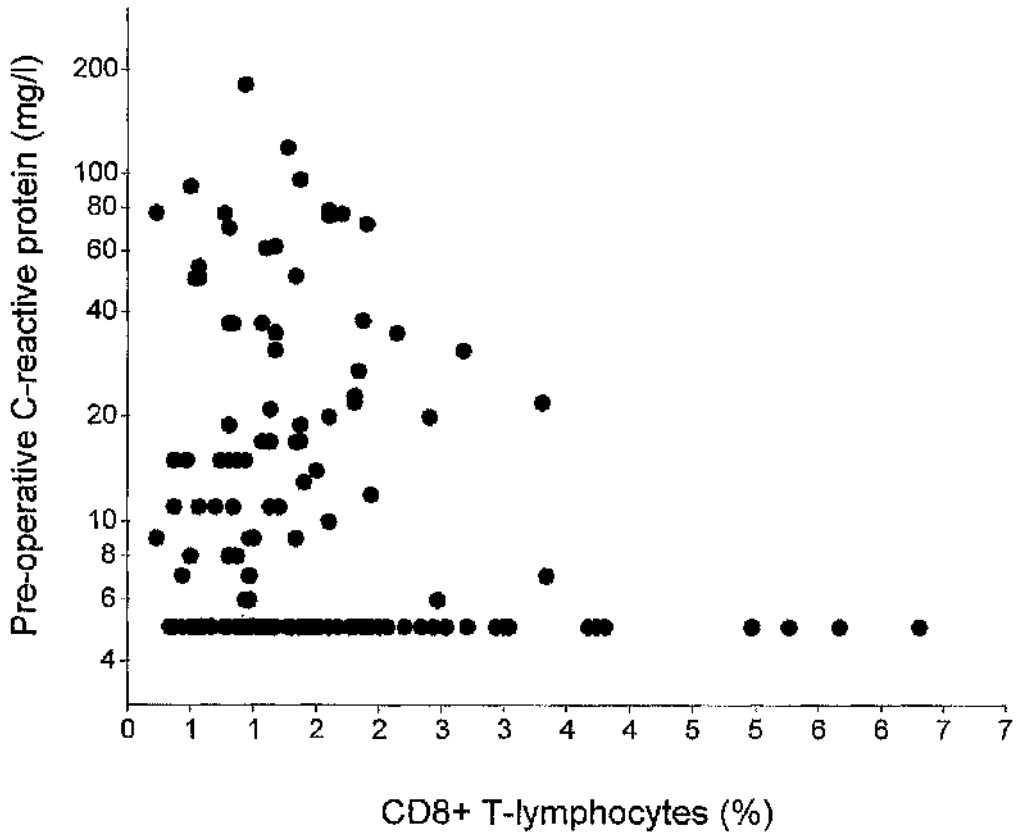


Figure 5.2 **Relationship between percentage tumour CD8+ T-lymphocyte infiltration and preoperative C-reactive protein in patients undergoing potentially curative resection for colorectal cancer.**

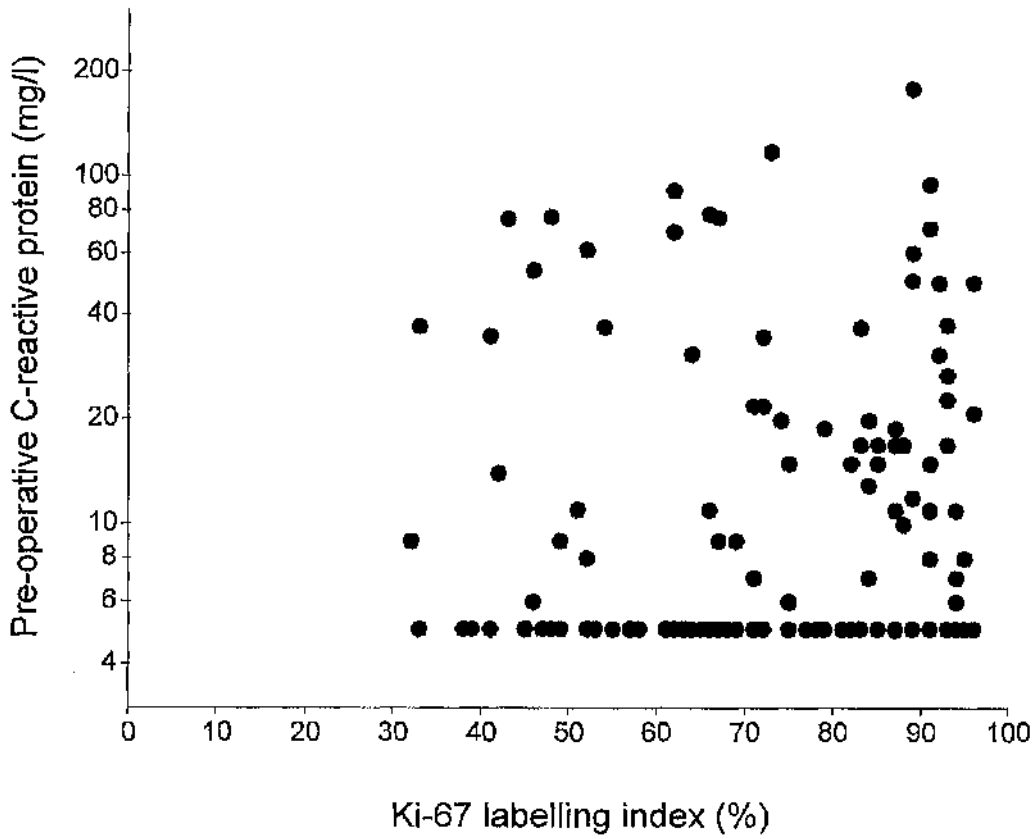


Figure 5.3 **Relationship between the tumour Ki-67 labelling index and preoperative C-reactive protein in patients undergoing potentially curative resection for colorectal cancer.**

CHAPTER SIX

6.0 CONCLUSION

Systemic inflammatory response and colorectal cancer:

The field of cancer research has largely been guided by a reductionist focus on cancer cells and the genes within them. This focus has produced an extraordinary body of knowledge but with little clinical impact. However, some workers have proposed that in the future important new developments in the treatment of cancer will come regarding tumours as complex tissues. Indeed, Hanahan and Weinberg (2000) have proposed that "important new inroads will come from regarding tumors as complex tissues in which mutant cancer cells have conscripted and subverted normal cell types to serve as active collaborators in their neoplastic agenda. The interactions between the genetically altered malignant cells and these supporting coconspirators will prove critical to understanding cancer pathogenesis and to the development of novel, effective therapies."

The present study examined, for the first time, the relationship between the pre-operative systemic inflammatory response and tumour based factors and suggests that the systemic inflammatory response is more closely related to outcome in patients undergoing potentially curative resection for colorectal cancer. One possible explanation is that C-reactive protein can be measured with greater accuracy and precision than tumour based factors.

Alternatively, it may be that C-reactive protein plays a more pivotal role in the tumour-host relationship. C-reactive protein is recognised to be an activator of innate immunity and a modulator of adaptive immunity (Du Clos and Mold, 2004). Its elevation is a

precursor to progressive involuntary loss of weight and lean tissue through an increase in the demand for certain amino acids, which if limited in the diet, may be obtained from the breakdown of skeletal muscle. In these ways the presence and magnitude of a chronic systemic inflammatory response may produce a progressive nutritional and functional decline ultimately resulting in reduced survival in the cancer (McMillan et al., 1998, Kotler, 2000).

The results of the present study do not exclude non-malignant causes of an elevated C-reactive protein in patients with colorectal cancer. However, it is of interest that C-reactive protein concentrations above the threshold used in the present study (>10mg/l) are rare (<5%) in the general elderly population in the West of Scotland (O'Reilly et al., 2006). However, it was of interest that, in the present study, an elevated C-reactive protein concentration prior to surgery, was associated with older age but not Dukes stage. This raises the question of whether those patients with an elevated C-reactive protein, prior to surgery had worse nutritional status or increased co-morbidity. Although we did not record nutritional status or co-morbidity in the present study it would be important to examine their relationship with the systemic inflammatory response in future studies since it may shed light on whether a poorer health state results in the tumour behaving more aggressively since this would be an important consideration in the administration of adjuvant chemotherapy.

The results of the present study are consistent with recent work which has shown that an elevated C-reactive protein concentration has prognostic value, independent of TNM stage, following resection, in variety of sold tumours (Ikeda et al., 2003; Jamieson et al., 2005; Lamb et al., 2006; Crumley et al., 2006). In the present study, when C-reactive

protein concentrations were combined with Dukes stage to form a new prognostic score, the combined score improved the prediction of cancer specific survival. The addition of C-reactive protein differentiated between low and high risk Dukes B and low and high risk Dukes C patients. Cancer related mortality ranged from 5% in patients with Dukes B tumours and a normal C-reactive protein concentration to 65% in patients with Dukes C tumours and an elevated C-reactive protein concentration. Cancer related mortality in patients with Dukes B tumours and an elevated C-reactive protein concentration was similar to that of patients with Dukes C tumours and a normal C-reactive protein concentration. However, the tumours in the present thesis were not pathologically staged according to TNM classification. Therefore it would be important to carry out further work in patients whose tumours were TNM staged. In particular, since surgery alone is the recommended treatment modality for TNM stage I/ II disease it would be important to confirm the prognostic value of a pre-operative C-reactive protein concentration in this node negative group.

Cellular Immunity and Colorectal Cancer

Cellular immunity is initiated by specifically sensitised T lymphocytes. It includes the classic delayed-type hypersensitivity reactions initiated by CD4+ T lymphocytes and direct cell cytotoxicity mediated by CD8+ T lymphocytes. The latter immune mechanism is important for the elimination of virally infected cells and tumour cells that express neoantigens.

In the present study, confined to patients with Dukes B and C colorectal cancer undergoing potentially curative resection, a number of factors were associated with poor cancer specific survival. In particular, a poor tumour CD4+ and CD8+ T-lymphocyte

infiltrate, an increased Ki-67 labelling index were associated with poorer cancer specific survival. Poor tumour CD4+ T-lymphocyte infiltrate was associated with an elevated circulating C-reactive protein and haemoglobin concentration but not tumour characteristics. Similarly, increased Ki-67 labelling index was associated with an elevated circulating C-reactive protein, tumour diameter but not other tumour characteristics. An elevated C-reactive protein, in addition to being associated with poor tumour CD4+ T-lymphocyte infiltrate and increased Ki-67 labelling index was also associated with tumour diameter. Taken together these results would suggest that local and systemic inflammatory responses are linked to tumour proliferation in patients with colorectal cancer and these responses are in turn linked to cancer specific survival, independent of tumour stage.

However, there are other aspects of the local inflammatory response that may be important and worthy of future study. In particular, the inter-relationships between tumour T-lymphocyte subsets infiltration, macrophage infiltration, microvessel density and COX-2 expression would also be of considerable interest since these are central to tumour dissemination and the local inflammatory process in the tumour respectively. Finally, it would be important to establish reliable methodology for the staining of interleukin-6 in paraffin-embedded colorectal tumours.

In summary, the results of the present thesis show that both an increased tumour Ki-67 labelling index and a low CD8+ T-lymphocyte count are independently associated with poorer outcome in patients undergoing curative resection for colorectal cancer. Given that C-reactive protein, a mediator of the innate immune response, had significant

prognostic value. These results may suggest that innate rather than cell mediated immunity is important in determining survival in colorectal cancer

Future work

The work in the present thesis suggest a number of avenues for research.

These include;

Confirming the prognostic value of a pre-operative C-reactive protein concentration in other centres, evaluating its value within the context of randomised adjuvant trials, comparing its prognostic value with other blood markers such as CEA and with more rigourous pathological criteria such as the proportion of positive lymph nodes.

Establishing the underlying basis of an elevated C-reactive protein concentration. It will be important to examine the relationship between C-reactive protein and tumour infiltration of inflammatory cells, other than T-lymphocytes of acquired immunity. These include macrophages, of the innate immune system, since they are known to release cytokines such as interleukin-6, interleukin-10 and vascular endothelial growth factor which are now recognised to stimulate tumour cell growth, inhibit T-lymphocytic function and promote angiogenesis respectively.

With such knowledge we will be in a position to devise rational strategies to moderate the systemic inflammatory response prior to surgery. If on reduction of the systemic inflammatory response, as evidenced by C-reactive protein, prior to surgery we are able to reduce tumour recurrence and prolong disease free survival in patients undergoing potentially curative resection for colorectal cancer, this would a major step forward in the treatment of primary colorectal cancer.

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APPENDIX: DATA FROM THE PROSPECTIVE STUDY FOR PATIENTS WITH OPERABLE COLORECTAL CANCER DESCRIBED IN CHAPTER FOUR AND FIVE.

APPENDIX: DATA FROM THE PROSPECTIVE STUDY FOR PATIENTS WITH OPERABLE COLORECTAL CANCER DESCRIBED IN CHAPTER FOUR AND FIVE.

Patient No	Age (Years)	Age code (<65=0, 65-74= 1 ≥75=2)	Sex (M=0,F=1)	Site (Rectum=0,colon=1)	Stage (Dukes B=0, C= 1)	Date of Operation	Type of operation	Cause of Death	Survival (months)
1	68	1.00	.00	.00	.00	09-Jan-1997	Ant.resection	2.00	59.47
2	46	.00	.00	.00	1.00	10-Jan-1997	Ant.resection	.00	97.07
3	56	.00	.00	1.00	.00	17-Jan-1997	Lt. Hemicoloectomy	.00	96.83
4	78	2.00	1.00	.00	.00	28-Jan-1997	Ant.resection	.00	96.47
5	76	2.00	.00	.00	.00	02-Feb-1997	Others	2.00	10.67
6	56	.00	.00	1.00	1.00	04-Feb-1997	Rt.Hemicoloectomy	.00	96.23
7	85	2.00	.00	1.00	1.00	05-Feb-1997	subtotal Coloectomy	1.00	31.97
8	75	2.00	.00	1.00	.00	14-Feb-1997	Rt.Hemicoloectomy	.00	95.90
9	81	2.00	1.00	1.00	.00	14-Feb-1997	Rt.Hemicoloectomy	1.00	7.57
10	77	2.00	1.00	1.00	1.00	21-Feb-1997	Rt.Hemicoloectomy	1.00	6.10
11	73	1.00	1.00	.00	1.00	04-Mar-1997	Ant.resection	.00	95.30
12	57	.00	.00	1.00	.00	06-Mar-1997	Sigmoid coloectomy	.00	95.23
13	90	2.00	1.00	1.00	.00	07-Mar-1997	Lt. Hemicoloectomy	2.00	44.23
14	61	.00	.00	.00	.00	13-Mar-1997	Ant.resection	.00	95.00
15	59	.00	1.00	1.00	.00	17-Mar-1997	Rt.Hemicoloectomy	.00	94.87
16	60	.00	1.00	1.00	.00	04-Apr-1997	Rt.Hemicoloectomy	.00	94.27
17	52	.00	.00	1.00	.00	08-Apr-1997	Sigmoid coloectomy	.00	94.13
18	79	2.00	1.00	.00	1.00	11-Apr-1997	Ant.resection	2.00	28.80
19	64	.00	.00	1.00	.00	13-Apr-1997	subtotal Coloectomy	1.00	88.27
21	66	1.00	.00	1.00	.00	22-Apr-1997	Sigmoid coloectomy	2.00	41.90
20	65	1.00	.00	1.00	.00	22-Apr-1997	subtotal Coloectomy	.00	93.67
22	66	1.00	.00	1.00	.00	25-Apr-1997	Rt.Hemicoloectomy	.00	93.57
23	78	2.00	.00	1.00	.00	29-Apr-1997	Rt.Hemicoloectomy	.00	93.43
24	62	.00	1.00	1.00	1.00	02-May-1997	Sigmoid coloectomy	.00	93.33
25	56	.00	1.00	1.00	.00	27-May-1997	Sigmoid coloectomy	1.00	52.73
26	43	.00	.00	1.00	.00	29-May-1997	Rt.Hemicoloectomy	.00	92.43
27	73	1.00	1.00	1.00	1.00	30-May-1997	Rt.Hemicoloectomy	1.00	17.30
28	77	2.00	1.00	1.00	1.00	24-Jun-1997	Rt.Hemicoloectomy	1.00	2.63
29	63	.00	1.00	1.00	1.00	25-Jun-1997	Sigmoid coloectomy	00	91.53
30	77	2.00	1.00	1.00	1.00	01-Jul-1997	Rt.Hemicoloectomy	1.00	16.47
31	71	1.00	1.00	1.00	.00	11-Jul-1997	Rt.Hemicoloectomy	.00	91.00
32	55	.00	1.00	.00	1.00	11-Jul-1997	Ant.resection	.00	91.00
33	78	2.00	1.00	1.00	1.00	18-Jul-1997	Rt.Hemicoloectomy	1.00	20.53

Patient No	Age (years)	Age code (<65=0, 65-74= 1 ≥75=2)	Sex (M=0,F=1)	Site (Rectum=0,colon=1)	Stage (Dukes B=0, C= 1)	D ate of Operation	Type of Operation	Cause of Death	Survival (Months)
34	51	.00	1.00	1.00	1.00	22-Jul-1997	Sigmoid colocctomy	.00	90.63
35	76	2.00	.00	.00	1.00	22-Jul-1997	Ant.resection	1.00	33.27
36	63	.00	.00	.00	1.00	24-Jul-1997	AP Resection	2.00	73.60
37	53	.00	1.00	1.00	.00	07-Aug-1997	Sigmoid colocctomy	.00	90.10
38	77	2.00	1.00	1.00	1.00	08-Aug-1997	Rt.Hemicoloectomy	1.00	17.07
39	79	2.00	.00	1.00	1.00	15-Aug-1997	Lt. Hemicoloectomy	1.00	10.67
40	67	1.00	.00	.00	.00	27-Aug-1997	Ant.resection	2.00	65.47
41	54	.00	1.00	1.00	1.00	16-Sep-1997	Rt.Hemicoloectomy	.00	88.77
42	47	.00	.00	1.00	1.00	25-Sep-1997	Rt.Hemicoloectomy	1.00	40.80
43	66	1.00	.00	.00	1.00	21-Oct-1997	Ant.resection	2.00	55.57
44	85	2.00	.00	1.00	.00	28-Nov-1997	Sigmoid colocctomy	1.00	48.70
45	86	2.00	.00	1.00	.00	04-Dec-1997	subtotal Colocctomy	2.00	45.20
46	73	1.00	1.00	.00	.00	16-Dec-1997	Ant.resection	.00	85.73
47	75	2.00	.00	1.00	.00	17-Dec-1997	Sigmoid colocctomy	2.00	47.13
48	54	.00	.00	1.00	.00	24-Dec-1997	subtotal Colocctomy	.00	85.47
49	75	2.00	1.00	1.00	1.00	30-Dec-1997	Lt. Hemicoloectomy	.00	85.27
50	75	2.00	1.00	1.00	.00	13-Feb-1998	Rt.Hemicoloectomy	.00	83.77
51	87	2.00	1.00	1.00	1.00	26-Feb-1998	Sigmoid colocctomy	1.00	26.97
52	73	1.00	.00	1.00	.00	25-Mar-1998	Rt.Hemicoloectomy	.00	82.43
53	89	2.00	.00	1.00	1.00	26-Mar-1998	Sigmoid colocctomy	1.00	27.87
54	78	2.00	.00	1.00	.00	12-Apr-1998	Sigmoid colocctomy	.00	81.83
55	78	2.00	.00	1.00	1.00	14-Apr-1998	Rt.Hemicoloectomy	1.00	11.77
56	70	1.00	.00	.00	.00	22-Apr-1998	AP Resection	.00	81.50
57	73	1.00	1.00	.00	.00	29-Apr-1998	Ant.resection	.00	81.27
58	83	2.00	1.00	.00	1.00	30-Apr-1998	Ant.resection	2.00	55.53
59	57	.00	.00	.00	.00	21-May-1998	Ant.resection	.00	80.53
60	71	1.00	1.00	.00	1.00	28-May-1998	Ant.resection	1.00	9.93
61	59	.00	.00	1.00	1.00	02-Jul-1998	Rt.Hemicoloectomy	.00	79.13
62	64	.00	.00	1.00	.00	06-Jul-1998	Rt.Hemicoloectomy	.00	79.00
63	89	2.00	1.00	.00	.00	09-Jul-1998	AP Resection	1.00	19.00
64	84	2.00	1.00	1.00	.00	14-Jul-1998	Rt.Hemicoloectomy	1.00	28.70
66	85	2.00	1.00	1.00	1.00	28-Jul-1998	Rt.Hemicoloectomy	1.00	7.83

Patient No	Age (Years)	Age code (<65=0, 65-74= 1 ≥75=2)	Sex (M=0,F=1)	Site (Rectum=0,colon=1)	Stage (Dukes B=0, C= 1)	Date of Operation	Type of operation	Cause of Death	Survival (months)
67	70	1.00	.00	.00	1.00	31-Jul-1998	Ant.resection	1.00	67.60
68	74	1.00	1.00	.00	1.00	25-Aug-1998	AP Resection	1.00	16.17
69	59	.00	.00	1.00	.00	27-Aug-1998	Sigmoid coloectomy	1.00	39.20
70	83	2.00	1.00	1.00	1.00	28-Aug-1998	Rt.Hemicoloectomy	1.00	19.00
71	42	.00	.00	1.00	.00	21-Sep-1998	Rt.Hemicoloectomy	.00	76.43
72	88	2.00	1.00	1.00	.00	28-Sep-1998	Sigmoid coloectomy	2.00	37.43
73	94	2.00	1.00	.00	.00	23-Oct-1998	Ant.resection	1.00	32.27
74	63	.00	.00	1.00	1.00	03-Nov-1998	Rt.Hemicoloectomy	1.00	36.23
75	66	1.00	1.00	.00	.00	05-Nov-1998	AP Resection	.00	74.93
76	82	2.00	1.00	.00	.00	20-Nov-1998	Ant.resection	.00	74.43
77	76	2.00	1.00	1.00	.00	24-Nov-1998	Sigmoid coloectomy	.00	74.30
78	76	2.00	.00	1.00	1.00	03-Dec-1998	Sigmoid coloectomy	.00	74.00
79	69	1.00	.00	1.00	1.00	07-Dec-1998	Sigmoid coloectomy	1.00	62.00
80	48	.00	.00	1.00	1.00	17-Dec-1998	Rt.Hemicoloectomy	.00	73.53
81	84	2.00	1.00	1.00	.00	29-Dec-1998	Lt. Hemicoloectomy	1.00	52.53
82	72	1.00	.00	1.00	.00	12-Jan-1999	Rt.Hemicoloectomy	.00	72.67
83	73	1.00	.00	1.00	.00	13-Jan-1999	Rt.Hemicoloectomy	.00	72.63
84	86	2.00	1.00	1.00	.00	22-Jan-1999	Sigmoid coloectomy	2.00	62.53
85	76	2.00	.00	1.00	1.00	01-Feb-1999	Rt.Hemicoloectomy	1.00	20.63
86	74	1.00	.00	1.00	.00	10-Feb-1999	Rt.Hemicoloectomy	.00	71.70
87	63	.00	1.00	1.00	.00	17-Feb-1999	Rt.Hemicoloectomy	.00	71.47
88	73	1.00	1.00	1.00	.00	25-Feb-1999	Rt.Hemicoloectomy	.00	71.20
89	65	1.00	1.00	.00	1.00	04-Mar-1999	Ant.resection	1.00	9.37
90	81	2.00	1.00	1.00	.00	15-Mar-1999	Rt.Hemicoloectomy	2.00	66.17
91	41	.00	.00	.00	1.00	16-Mar-1999	Ant.resection	.00	70.57
92	65	1.00	1.00	1.00	.00	17-Mar-1999	Sigmoid coloectomy	.00	70.53
93	77	2.00	1.00	1.00	.00	08-May-1999	subtotal Coloectomy	1.00	6.90
94	60	.00	1.00	1.00	.00	21-May-1999	Sigmoid coloectomy	.00	68.37
95	78	2.00	.00	1.00	.00	18-Jun-1999	Lt. Hemicoloectomy	2.00	51.70
96	60	.00	.00	.00	.00	23-Jun-1999	Ant.resection	.00	67.27
97	73	1.00	.00	1.00	.00	29-Jun-1999	Sigmoid coloectomy	.00	67.07
98	59	.00	.00	1.00	1.00	13-Jul-1999	Rt.Hemicoloectomy	.00	66.60
99	78	2.00	1.00	1.00	.00	23-Jul-1999	Lt. Hemicoloectomy	1.00	27.13

Patient No	Age (Years)	Age code (<65=0, 65-74= 1 ≥75=2)	Sex (M=0,F=1)	Site (Rectum=0,colon=1)	Stage (Dukes B=0, C= 1)	Date of Operation	Type of operation	Cause of Death	Survival (months)
100	84	2.00	1.00	1.00	1.00	05-Aug-1999	Rt.Hemicoloectomy	.00	65.83
101	84	2.00	1.00	.00	.00	15-Aug-1999	Ant.resection	.00	65.50
102	66	1.00	1.00	.00	.00	18-Aug-1999	Ant.resection	.00	65.40
103	98	2.00	1.00	.00	1.00	24-Aug-1999	Ant.resection	1.00	26.47
104	87	2.00	.00	1.00	.00	08-Sep-1999	Rt.Hemicoloectomy	.00	64.70
105	78	2.00	.00	.00	.00	15-Sep-1999	Ant.resection	.00	64.47
106	65	1.00	1.00	1.00	.00	24-Sep-1999	Rt.Hemicoloectomy	.00	64.17
107	70	1.00	.00	1.00	1.00	05-Oct-1999	Sigmoid coloectomy	.00	63.80
108	60	.00	1.00	1.00	.00	08-Oct-1999	Sigmoid coloectomy	.00	63.70
109	60	.00	1.00	1.00	.00	21-Oct-1999	Rt.Hemicoloectomy	.00	63.27
110	72	1.00	.00	.00	1.00	22-Oct-1999	Ant.resection	.00	63.23
111	66	1.00	1.00	.00	1.00	27-Oct-1999	Ant.resection	.00	63.07
112	56	.00	.00	.00	1.00	18-Nov-1999	AP Resection	2.00	40.67
113	78	2.00	.00	1.00	.00	25-Nov-1999	Rt.Hemicoloectomy	.00	62.10
114	82	2.00	1.00	1.00	1.00	25-Nov-1999	Rt.Hemicoloectomy	1.00	43.33
115	69	1.00	.00	1.00	1.00	30-Nov-1999	subtotal Colectomy	1.00	35.50
116	72	1.00	.00	.00	.00	08-Dec-1999	Ant.resection	.00	61.67
117	72	1.00	.00	1.00	.00	16-Dec-1999	Rt.Hemicoloectomy	.00	61.40
118	81	2.00	1.00	1.00	.00	26-Jan-2000	Rt.Hemicoloectomy	.00	60.03
119	66	1.00	.00	1.00	.00	03-Mar-2000	Sigmoid coloectomy	1.00	42.37
120	45	.00	1.00	1.00	.00	08-Mar-2000	Rt.Hemicoloectomy	.00	58.63
121	79	2.00	1.00	1.00	.00	15-Mar-2000	Rt.Hemicoloectomy	2.00	30.37
122	63	.00	.00	1.00	1.00	25-Jul-2000	Rt.Hemicoloectomy	1.00	20.03
123	89	2.00	1.00	1.00	1.00	08-Sep-2000	Rt.Hemicoloectomy	2.00	33.30
124	82	2.00	1.00	1.00	.00	20-Sep-2000	Rt.Hemicoloectomy	.00	52.10
125	70	1.00	.00	1.00	.00	27-Oct-2000	Sigmoid coloectomy	.00	50.87
126	56	.00	.00	1.00	.00	09-Nov-2000	Sigmoid coloectomy	.00	50.43
127	70	1.00	.00	.00	.00	05-Dec-2000	Ant.resection	.00	49.57
128	64	.00	.00	1.00	1.00	14-Dec-2000	Rt.Hemicoloectomy	.00	49.27
129	78	2.00	.00	1.00	.00	16-Jan-2001	Sigmoid coloectomy	.00	48.17
130	72	1.00	.00	.00	.00	24-Jan-2001	Ant.resection	.00	47.90
131	68	1.00	.00	1.00	.00	24-Jan-2001	subtotal Colectomy	1.00	13.77
132	64	.00	.00	1.00	1.00	30-Jan-2001	Rt.Hemicoloectomy	.00	47.70

Patient No	Age (Years)	Age code (<65=0, 65-74= 1 ≥75=2)	Sex (M=0,F=1)	Site (Rectum=0,colon=1)	Stage (Dukes B=0, C= 1)	Date of Operation	Type of operation	Cause of Death	Survival (months)
133	72	1.00	.00	1.00	.00	31-Jan-2001	Rt.Hemicolocctomy	.00	47.67
134	55	.00	.00	1.00	.00	14-Feb-2001	Sigmoid colectomy	.00	47.20
135	83	2.00	.00	1.00	.00	09-Mar-2001	Rt.Hemicolectomy	.00	46.43
137	55	.00	.00	1.00	1.00	13-Mar-2001	Rt.Hemicolectomy	.00	46.30
136	57	.00	1.00	.00	.00	13-Mar-2001	Ant.resection	.00	46.30
138	42	.00	1.00	1.00	.00	03-May-2001	Rt.Hemicolectomy	.00	44.60
139	60	.00	1.00	.00	1.00	07-May-2001	Others	1.00	9.87
140	71	1.00	1.00	1.00	.00	08-May-2001	Sigmoid colectomy	.00	44.43
141	79	2.00	.00	1.00	1.00	12-Jul-2001	Rt.Hemicolectomy	.00	42.27
142	40	.00	.00	1.00	.00	19-Jul-2001	Rt.Hemicolectomy	.00	42.03
143	73	1.00	1.00	1.00	.00	21-Aug-2001	subtotal Colectomy	.00	40.93
144	69	1.00	.00	1.00	.00	23-Aug-2001	subtotal Colectomy	.00	40.87
145	70	1.00	.00	.00	1.00	04-Sep-2001	Ant.resection	.00	40.47
146	75	2.00	1.00	1.00	.00	06-Sep-2001	Rt.Hemicolectomy	.00	40.40
147	82	2.00	1.00	.00	.00	27-Sep-2001	Ant.resection	2.00	34.90

Age code: <65=0, 65-74= 1, ≥75=2; Sex M = 0, F= 1; Site colon= 1, rectum= 0; Dukes stage B= 0, C= 1; Cause of death cancer related= 1, non-cancer related= 2.

Patient No	Preoperative C-reactive Protein (mg/l)	C-reactive protein (≤10mg/l=0/ >10mg/l=1)	Albumin (g/l)	Tumour Ki–67	Tumour KI-67 (tertiles)	Tumour CD4+ T-lymphocyte (tertiles)	Tumour CD4+ T-lymphocyte percentage	Tumour CD48+ T-lymphocyte (tertiles)	Tumour CD8+ T-lymphocyte percentage
1	5.00	.00	42.00	.72	1.00	28.00	.01	58.00	.02
2	6.00	.00	40.00	.46	.00	37.00	.01	29.00	.01
3	5.00	.00	41.00	.55	.00	18.00	.01	31.00	.01
4	5.00	.00	36.00	.58	.00	11.00	.00	43.00	.01
5	5.00	.00	35.00	.87	2.00	53.00	.02	13.00	.00
6	7.00	.00	40.00	.84	1.00	58.00	.02	100.00	.03
7	15.00	1.00	39.00	.82	1.00	16.00	.01	24.00	.01
8	20.00	1.00	43.00	.84	1.00	47.00	.02	72.00	.02
9	54.00	1.00	36.00	.46	.00	17.00	.01	17.00	.01
10	13.00	1.00	30.00	.84	1.00	34.00	.01	42.00	.01
11	5.00	.00	36.00	.96	2.00	26.00	.01	58.00	.02
12	5.00	.00	36.00	.69	1.00	20.00	.01	27.00	.01
13	51.00	1.00	27.00	.89	2.00	19.00	.01	40.00	.01
14	5.00	.00	33.00	.79	1.00	35.00	.01	26.00	.01
15	70.00	1.00	39.00	.62	.00	5.00	.00	24.00	.01
16	37.00	1.00	34.00	.33	.00	16.00	.01	24.00	.01
17	5.00	.00	40.00	.58	.00	39.00	.01	54.00	.02
18	27.00	1.00	31.00	.93	2.00	17.00	.01	55.00	.02
19	5.00	.00	31.00	.87	2.00	30.00	.01	17.00	.01
21	20.00	1.00	42.00	.74	1.00	40.00	.01	48.00	.02
20	5.00	.00	39.00	.89	2.00	25.00	.01	110.00	.04
22	7.00	.00	41.00	.71	1.00	13.00	.00	29.00	.01
23	5.00	.00	40.00	.65	1.00	12.00	.00	28.00	.01
24	5.00	.00	39.00	.45	.00	41.00	.01	81.00	.03
25	9.00	.00	41.00	.67	1.00	35.00	.01	7.00	.00
26	5.00	.00	38.00	.58	.00	42.00	.01	23.00	.01
27	5.00	.00	31.00	.93	2.00	12.00	.00	24.00	.01
28	5.00	.00	37.00	.61	.00	69.00	.02	54.00	.02
29	5.00	.00	38.00	.87	2.00	52.00	.02	23.00	.01
30	10.00	.00	40.00	.88	2.00	67.00	.02	48.00	.02

Patient No	Preoperative C-reactive Protein (mg/l)	C-reactive protein (≤10mg/l=0/ >10mg/l=1)	Albumin (g/l)	Tumour Ki-67	Tumour KI-67 (tertiles)	Tumour CD4+ T-lymphocyte (tertiles)	Tumour CD4+ T-lymphocyte percentage	Tumour CD48+ T-lymphocyte (tertiles)	Tumour CD8+ T-lymphocyte percentage
31	5.00	.00	35.00	.91	2.00	30.00	.01	43.00	.01
32	17.00	1.00	37.00	.88	2.00	29.00	.01	32.00	.01
33	35.00	1.00	42.00	.72	1.00	38.00	.01	35.00	.01
34	5.00	.00	42.00	.58	.00	24.00	.01	20.00	.01
35	19.00	1.00	32.00	.87	2.00	21.00	.01	41.00	.01
36	5.00	.00	43.00	.65	.00	32.00	.01	25.00	.01
37	5.00	.00	36.00	.67	1.00	19.00	.01	29.00	.01
38	14.00	1.00	29.00	.42	.00	19.00	.01	45.00	.02
39	5.00	.00	37.00	.89	2.00	17.00	.01	53.00	.02
40	5.00	.00	42.00	.68	1.00	55.00	.02	50.00	.02
41	5.00	.00	42.00	.82	1.00	41.00	.01	73.00	.02
42	15.00	1.00	34.00	.91	2.00	10.00	.00	11.00	.00
43	5.00	.00	42.00	.78	1.00	12.00	.00	11.00	.00
44	17.00	1.00	35.00	.83	1.00	27.00	.01	41.00	.01
45	5.00	.00	41.00	.52	.00	50.00	.02	46.00	.02
46	5.00	.00	38.00	.66	1.00	14.00	.00	18.00	.01
47	15.00	1.00	33.00	.75	1.00	15.00	01	14.00	.00
48	5.00	.00	37.00	.89	2.00	39.00	.01	41.00	.01
49	5.00	.00	46.00	.83	1.00	57.00	.02	38.00	.01
50	5.00	.00	33.00	.66	1.00	23.00	.01	38.00	.01
51	11.00	1.00	36.00	.91	2.00	8.00	.00	25.00	.01
52	8.00	.00	40.00	.52	.00	21.00	.01	15.00	.01
53	11.00	1.00	20.00	.51	2.00	18.00	.01	17.00	.01
54	19.00	1.00	40.00	.79	1.00	23.00	.01	24.00	.01
55	11.00	1.00	39.00	.51	.00	25.00	.01	34.00	.01
56	5.00	.00	41.00	.67	1.00	23.00	.01	170.00	.06
57	5.00	.00	42.00	.38	.00	36.00	.01	66.00	.02
58	5.00	.00	39.00	.39	.00	23.00	.01	45.00	.02
59	15.00	1.00	31.00	.91	2.00	15.00	.01	28.00	.01
60	11.00	1.00	43.00	.68	1.00	12.00	.00	21.00	.01

Patient No	Preoperative C-reactive Protein (mg/l)	C-reactive protein (≤10mg/l=0/ >10mg/l=1)	Albumin (g/l)	Tumour Ki-67	Tumour KI-67 (tertiles)	Tumour CD4+ T-lymphocyte (tertiles)	Tumour CD4+ T-lymphocyte percentage	Tumour CD48+ T-lymphocyte (tertiles)	Tumour CD8+ T-lymphocyte percentage
61	5.00	.00	47.00	.47	.00	24.00	.01	30.00	.01
62	76.00	1.00	42.00	.43	.00	78.00	.03	48.00	.02
63	15.00	1.00	31.00	.91	2.00	20.00	.01	26.00	.01
64	50.00	1.00	26.00	.92	2.00	34.00	.01	16.00	.01
65	5.00	.00	42.00	.77	1.00	53.00	.02	60.00	.02
66	72.00	1.00	27.00	.91	2.00	4.00	.00	57.00	.02
67	5.00	.00	45.00	.57	.00	19.00	.01	11.00	.00
68	77.00	1.00	41.00	.48	.00	8.00	.00	7.00	.00
69	5.00	.00	45.00	.75	1.00	27.00	.01	32.00	.01
70	38.00	1.00	28.00	.93	2.00	21.00	.01	56.00	.02
71	9.00	.00	#NULL!	.32	.00	70.00	.02	29.00	.01
72	11.00	1.00	40.00	.87	2.00	20.00	.01	36.00	.01
73	12.00	1.00	29.00	.89	2.00	17.00	.01	58.00	.02
74	17.00	1.00	44.00	.93	2.00	61.00	.02	40.00	.01
75	5.00	.00	43.00	.94	2.00	47.00	.02	26.00	.01
76	5.00	.00	41.00	.87	2.00	13.00	.00	16.00	.01
77	5.00	.00	32.00	.48	.00	23.00	.01	33.00	.01
78	5.00	.00	35.00	.38	.00	71.00	.02	62.00	.02
79	62.00	1.00	40.00	.52	.00	44.00	.01	35.00	.01
80	5.00	.00	45.00	.67	1.00	59.00	.02	189.00	.06
81	5.00	.00	43.00	.61	.00	33.00	.01	38.00	.01
82	79.00	1.00	31.00	.66	1.00	10.00	.00	48.00	.02
83	22.00	1.00	37.00	.72	1.00	29.00	.01	99.00	.03
84	5.00	.00	41.00	.33	.00	6.00	.00	15.00	.01
85	5.00	.00	37.00	.85	1.00	8.00	.00	10.00	.00
86	5.00	.00	45.00	.53	.00	41.00	.01	38.00	.01
87	5.00	.00	38.00	.71	1.00	21.00	.01	44.00	.01
88	5.00	.00	42.00	.63	.00	49.00	.02	158.00	.05
89	15.00	1.00	33.00	.85	1.00	9.00	.00	22.00	.01
90	5.00	.00	36.00	.67	2.00	39.00	.01	112.00	.04

Patient No	Preoperative C-reactive Protein (mg/l)	C-reactive protein (≤10mg/l=0/ >10mg/l=1)	Albumin (g/l)	Tumour Ki-67	Tumour KI-67 (tertiles)	Tumour CD4+ T-lymphocyte (tertiles)	Tumour CD4+ T-lymphocyte percentage	Tumour CD48+ T-lymphocyte (tertiles)	Tumour CD8+ T-lymphocyte percentage
91	5.00	.00	43.00	.89	2.00	57.00	.02	76.00	.03
92	8.00	.00	41.00	.91	2.00	37.00	.01	24.00	.01
93	50.00	1.00	28.00	.96	2.00	3.00	.00	17.00	.01
94	5.00	.00	35.00	.64	.00	17.00	.01	27.00	.01
95	5.00	.00	40.00	.41	.00	2.00	.00	114.00	.04
96	5.00	.00	41.00	.58	.00	61.00	.02	32.00	.01
97	5.00	.00	39.00	.53	.00	14.00	.00	18.00	.01
98	9.00	.00	35.00	.69	1.00	54.00	.02	30.00	.01
99	17.00	1.00	31.00	.87	2.00	24.00	.01	32.00	.01
100	5.00	.00	37.00	.62	.00	60.00	.02	56.00	.02
101	35.00	1.00	39.00	.41	.00	42.00	.01	64.00	.02
102	5.00	.00	32.00	.52	.00	84.00	.03	48.00	.02
103	5.00	.00	40.00	.95	2.00	58.00	.02	42.00	.01
104	5.00	.00	40.00	.78	1.00	62.00	.02	149.00	.05
105	5.00	.00	43.00	.49	.00	48.00	.02	35.00	.01
106	5.00	.00	42.00	.39	.00	3.00	.00	26.00	.01
107	5.00	.00	36.00	.71	1.00	107.00	.04	57.00	.02
108	5.00	.00	35.00	.64	.00	23.00	.01	28.00	.01
109	61.00	1.00	39.00	.89	2.00	25.00	.01	33.00	.01
110	5.00	.00	46.00	.68	1.00	45.00	.02	28.00	.01
111	5.00	.00	38.00	.62	.00	49.00	.02	30.00	.01
112	5.00	.00	36.00	.61	.00	36.00	.01	29.00	.01
113	37.00	1.00	44.00	.83	1.00	34.00	.01	32.00	.01
114	23.00	1.00	38.00	.93	2.00	26.00	.01	54.00	.02
115	11.00	1.00	35.00	.94	2.00	42.00	.01	11.00	.00
116	5.00	.00	37.00	.65	1.00	59.00	.02	91.00	.03
117	5.00	.00	32.00	.64	.00	86.00	.03	27.00	.01
118	5.00	.00	33.00	.91	2.00	30.00	.01	70.00	.02
119	5.00	.00	31.00	.81	1.00	72.00	.02	27.00	.01
120	5.00	.00	41.00	.91	2.00	53.00	.02	90.00	.03

Patient No	Preoperative C-reactive Protein (mg/l)	C-reactive protein (≤10mg/l=0/ >10mg/l=1)	Albumin (g/l)	Tumour Ki-67	Tumour KI-67 (tertiles)	Tumour CD4+ T-lymphocyte (tertiles)	Tumour CD4+ T-lymphocyte percentage	Tumour CD48+ T-lymphocyte (tertiles)	Tumour CD8+ T-lymphocyte percentage
121	31.00	1.00	32.00	.64	.00	23.00	.01	35.00	.01
122	97.00	1.00	23.00	.91	2.00	91.00	.03	41.00	.01
123	31.00	1.00	39.00	.92	2.00	32.00	.01	80.00	.03
124	8.00	.00	42.00	.95	2.00	17.00	.01	26.00	.01
125	9.00	.00	30.00	.49	.00	82.00	.03	40.00	.01
126	77.00	1.00	38.00	.67	1.00	27.00	.01	23.00	.01
127	5.00	.00	35.00	.45	.00	30.00	.01	44.00	.01
128	5.00	.00	34.00	.58	.00	1.00	.00	23.00	.01
129	5.00	.00	32.00	.91	2.00	58.00	.02	88.00	.03
130	5.00	.00	34.00	.96	2.00	23.00	.01	28.00	.01
131	77.00	1.00	28.00	.67	1.00	41.00	.01	51.00	.02
132	5.00	.00	36.00	.95	2.00	25.00	.01	39.00	.01
133	5.00	.00	40.00	.71	1.00	18.00	.01	24.00	.01
134	5.00	.00	34.00	.78	1.00	38.00	.01	34.00	.01
135	17.00	1.00	34.00	.85	1.00	8.00	.00	34.00	.01
137	37.00	1.00	36.00	.54	.00	13.00	.00	25.00	.01
136	5.00	.00	33.00	.96	2.00	40.00	.02	30.00	.01
138	5.00	.00	35.00	.75	1.00	24.00	.01	18.00	.01
139	120.00	1.00	23.00	.73	1.00	7.00	.00	38.00	.01
140	6.00	.00	37.00	.94	2.00	54.00	.02	74.00	.02
141	92.00	1.00	39.00	.62	.00	15.00	.01	15 00	.01
142	182.00	1.00	41.00	.89	2.00	17.00	.01	28.00	.01
143	7.00	.00	31.00	.94	2.00	11.00	.00	13.00	.00
144	22.00	1.00	32.00	.71	1.00	43.00	.01	54.00	.02
145	5.00	.00	36.00	.71	1.00	49.00	.02	23.00	.01
146	6.00	.00	32.00	.75	1.00	34.00	.01	28.00	.01
147	21.00	1.00	41.00	.96	2.00	15.00	.01	34.00	.01

Pre-operative C-reactive protein ≤10mg/l = 0, >10mg/l = 1; Ki-67 labelling index code (%) 32-65 % = 0, 65-85 % = 1, 87-96 % = 2.

Patient No	Tumour CD 3+ T-lymphocyte percentage	WBC * 10^9/L	Neutrophil	Lymphocytes	Haemoglobulin (gm/dl)	Platelets * 10^9/L	Size (mm)	Size code (10-35mm = 0, 35-50mm = 1, 50-130mm= 2)
1	.03	5.40	2.80	1.50	13.90	200.00	50.00	2.00
2	.02	9.40	5.60	2.60	17.00	320.00	30.00	.00
3	.02	4.90	3.10	.90	11.00	271.00	50.00	1.00
4	.02	5.50	3.90	1.20	8.40	161.00	25.00	.00
5	.02	7.80	6.20	.90	12.80	340.00	40.00	1.00
6	.05	11.20	7.60	2.70	10.90	407.00	50.00	2.00
7	.01	5.20	4.20	.70	11.30	242.00	30.00	.00
8	.04	8.90	6.50	1.50	13.00	339.00	45.00	1.00
9	.01	11.10	10.00	.50	12.10	465.00	45.00	1.00
10	.03	11.50	7.80	2.40	11.30	396.00	45.00	1.00
11	.03	11.00	6.60	2.90	12.40	343.00	55.00	2.00
12	.02	12.40	11.30	.40	9.00	306.00	90.00	2.00
13	.02	17.90	16.40	.50	9.80	261.00	80.00	2.00
14	.02	7.60	4.40	2.30	11.80	223.00	70.00	2.00
15	.01	17.00	12.20	3.40	13.00	451.00	60.00	2.00
16	.01	8.20	4.60	2.30	10.80	520.00	30.00	.00
17	.03	8.00	6.40	1.20	13.30	227.00	60.00	2.00
18	.02	16.70	14.50	1.50	11.20	382.00	40.00	1.00
19	.02	7.30	6.20	.80	13.70	219.00	25.00	.00
21	.03	7.50	5.50	1.00	12.40	249.00	35.00	.00
20	.05	7.10	7.70	1.20	12.80	315.00	28.00	.00
22	.01	5.40	3.70	1.00	10.60	327.00	50.00	1.00
23	.01	8.40	5.50	1.80	11.50	317.00	50.00	1.00
24	.04	6.80	4.50	1.40	11.50	108.00	30.00	.00
25	.01	7.80	6.80	.50	10.70	124.00	22.00	.00
26	.02	15.40	14.00	.90	9.80	651.00	35.00	.00
27	.01	7.50	5.60	1.40	11.10	285.00	60.00	2.00
28	.04	8.00	6.40	1.30	10.30	155.00	22.00	.00
29	.03	6.30	4.60	1.10	14.20	192.00	30.00	.00
30	.04	8.80	6.80	1.20	10.70	289.00	20.00	.00

Patient No	Tumour CD 3+ T-lymphocyte percentage	WBC * 10^9/L	Neutrophil	Lymphocytes	Haemoglobulin (gm/dl)	Platelets * 10^9/L	Size (mm)	Size code (10-35mm = 0, 35-50mm = 1, 50-130mm= 2)
31	.02	5.70	3.80	1.20	9.60	396.00	50.00	2.00
32	.02	8.30	5.40	2.60	13.60	232.00	48.00	1.00
33	.02	7.10	5.20	1.40	12.90	262.00	40.00	1.00
34	.01	6.60	4.20	1.70	11.40	305.00	60.00	2.00
35	.02	5.00	4.10	.40	12.80	261.00	60.00	2.00
36	.02	9.90	8.40	.90	11.80	508.00	35.00	.00
37	.02	13.50	12.90	.20	11.10	229.00	30.00	.00
38	.02	8.50	7.10	1.00	11.80	226.00	30.00	.00
39	.02	7.10	3.60	2.50	15.00	174.00	30.00	.00
40	.04	7.30	6.50	.50	12.40	177.00	25.00	.00
41	.04	12.20	8.40	2.90	11.90	53.00	60.00	2.00
42	.01	7.70	5.00	2.00	12.00	204.00	70.00	2.00
43	.01	12.00	9.50	1.40	13.60	372.00	50.00	2.00
44	.02	7.90	5.70	1 30	11.00	254.00	50.00	2.00
45	.03	4.80	2.90	1.40	12.20	144.00	20.00	.00
46	.01	7.00	4 10	2.10	13.50	401.00	50.00	1.00
47	.01	8.60	7.90	.30	11.90	281.00	50.00	2.00
48	.03	11.20	9.50	1.30	14.00	263.00	35.00	1.00
49	.03	6.10	3.90	1.60	13.10	203.00	40.00	1.00
50	.02	12.40	6.80	4.60	12.20	390.00	120.00	2.00
51	.01	7.80	5.90	1.00	12.00	196.00	30.00	.00
52	.01	5.80	3.70	1.40	11.10	351.00	40.00	1.00
53	.01	8.40	5.90	1.90	12.60	240.00	20.00	.00
54	.02	14.40	12.10	1.40	10.80	239.00	70.00	2.00
55	.02	6.70	4.20	1.30	11.90	298.00	40.00	1.00
56	.06	12.10	10.00	1.20	10.40	174.00	70.00	2.00
57	.03	7.90	4.70	2.50	12.50	232.00	35.00	.00
58	.02	7.40	4.40	2.20	10.70	198.00	35.00	.00
59	.01	8.70	5.50	2.50	14.70	195.00	45.00	1.00
60	.01	5.80	3.90	1.50	11.80	200.00	50.00	1.00

Patient No	Tumour CD 3+ T-lymphocyte percentage	WBC * 10^9/L	Neutrophil	Lymphocytes	Haemoglobulin (gm/dl)	Platelets * 10^9/L	Size (mm)	Size code (10-35mm = 0, 35-50mm = 1, 50-130mm = 2)
61	.02	8.80	5.60	2.00	12.00	352.00	28.00	.00
62	.04	4.50	2.90	.90	12.50	240.00	40.00	1.00
63	.02	13.60	12.40	.70	10.80	275.00	50.00	2.00
64	.02	7.30	4.40	2.10	11.20	291.00	70.00	2.00
65	.04	7.10	4.60	1.80	10.00	282.00	20.00	.00
66	.02	10.70	8.70	1.20	11.20	347.00	70.00	2.00
67	.01	7.70	5.00	2.10	15.30	214.00	25.00	.00
68	.01	6.80	4.40	1.70	13.50	272.00	40.00	1.00
69	.02	7.90	4.30	2.70	13.50	351.00	30.00	.00
70	.03	9.80	6.40	2.40	11.20	486.00	60.00	2.00
71	.03	17.60	14.70	1.50	12.50	263.00	30.00	.00
72	.02	7.80	6.30	.90	9.10	267.00	65.00	2.00
73	.03	5.90	3.30	1.70	11.20	378.00	35.00	1.00
74	.03	5.90	3.30	2.00	14.60	267.00	25.00	.00
75	.02	6.40	4.30	1.60	12.90	329.00	25.00	.00
76	.01	5.70	4.10	1.20	11.00	265.00	55.00	2.00
77	.02	7.20	5.10	1.50	11.30	327.00	35.00	.00
78	.04	8.40	7.00	1.00	14.50	275.00	30.00	.00
79	.03	11.50	5.00	4.50	14.30	332.00	45.00	1.00
80	.08	8.20	6.50	1.20	12.40	329.00	55.00	2.00
81	.02	7.40	6.00	.80	14.20	183.00	40.00	1.00
82	.02	7.60	6.80	.60	10.60	342.00	57.00	2.00
83	.04	8.80	7.50	.80	10.40	379.00	55.00	2.00
84	.01	7.30	5.50	1.10	13.00	209.00	45.00	1.00
85	.01	8.30	7.40	.50	11.30	325.00	35.00	1.00
86	.03	8.30	5.70	1.90	12.20	432.00	40.00	1.00
87	.02	17.60	13.40	1.00	14.50	463.00	115.00	2.00
88	.07	11.40	7.10	3.30	8.90	604.00	95.00	2.00
89	.01	6.90	4.10	1.90	13.70	214.00	35.00	1.00
90	.05	3.30	2.00	1.00	9.80	206.00	30.00	.00

Patient No	Tumour CD 3+ T-lymphocyte percentage	WBC * 10^9/L	Neutrophil	Lymphocytes	Haemoglobulin (gm/dl)	Platelets * 10^9/L	Size (mm)	Size code (10-35mm = 0, 35-50mm = 1, 50-130mm= 2)
91	.04	7.20	6.40	.40	13.30	223.00	40.00	1.00
92	.02	7.60	4.80	1.90	13.30	308.00	35.00	1.00
93	.01	25.60	24.20	.70	9.90	240.00	130.00	2.00
94	.01	5.30	3.40	1.30	10.80	343.00	50.00	1.00
95	.04	8.40	5.30	2.10	11.70	389.00	45.00	1.00
96	.03	5.70	4.90	.30	11.00	142.00	15.00	.00
97	.01	6.70	4.00	1.80	14.70	171.00	40.00	1.00
98	.03	6.60	3.80	2.00	9.70	378.00	40.00	1.00
99	.02	7.70	4.90	1.90	9.20	497.00	35.00	1.00
100	.04	9.30	6.70	1.90	10.90	587.00	35.00	.00
101	.04	7.60	5.00	1.70	11.70	278.00	55.00	2.00
102	.04	7.30	5.50	1.30	15.20	191.00	40.00	1.00
103	.03	7.60	4.90	2.10	11.50	434.00	40.00	1.00
104	.07	8.90	5.40	2.30	12.00	355.00	50.00	2.00
105	.03	16.50	15.40	.30	11.60	244.00	35.00	.00
106	.01	7.90	3.40	3.60	9.70	364.00	35.00	.00
107	.05	8.40	4.60	2.90	13.90	242.00	40.00	1.00
108	.02	5.30	3.60	1.00	12.40	249.00	47.00	1.00
109	.02	9.60	7.50	1.20	9.30	499.00	55.00	2.00
110	.02	9.60	6.70	1.90	12.20	469.00	10.00	.00
111	.03	6.30	3.80	1.60	15.10	195.00	25.00	.00
112	.02	6.40	4.30	1.50	14.40	235.00	30.00	.00
113	.02	8.30	6.00	1.60	12.90	340.00	60.00	2.00
114	.03	10.40	6.20	3.30	10.10	351.00	35.00	1.00
115	.02	9.50	5.40	2.20	11.20	343.00	35.00	1.00
116	.05	4.90	2.10	1.90	12.90	267.00	55.00	2.00
117	.04	14.40	7.60	1.70	8.70	494.00	80.00	2.00
118	.03	10.30	5.40	4.00	12.20	362.00	16.00	.00
119	.03	5.30	2.90	1.40	13.70	199.00	25.00	.00
120	.05	10.20	6.80	2.10	12.80	390.00	50.00	2.00

Patient No	Tumour CD 3+ T-lymphocyte percentage	WBC * 10^9/L	Neutrophil	Lymphocytes	Haemoglobulin (gm/dl)	Platelets * 10^9/L	Size (mm)	Size code (10-35mm = 0, 35-50mm = 1, 50-130mm= 2)
121	.02	5.90	4.50	.80	10.00	161.00	30.00	.00
122	.04	9.50	7.40	1.30	14.40	321.00	70.00	2.00
123	.04	10.90	9.10	.90	12.20	313.00	80.00	2.00
124	.01	8.20	6.70	.90	11.40	195.00	45.00	1.00
125	.04	7.00	5.00	1.20	11.30	531.00	30.00	.00
126	.02	12.30	9.70	1.80	8.60	668.00	55.00	2.00
127	.02	10.10	7.90	.80	10.80	230.00	50.00	1.00
128	.01	6.70	4.50	1.40	13.70	317.00	30.00	.00
129	.05	5.60	3.30	1.70	9.20	300.00	40.00	1.00
130	.02	9.00	8.00	.50	10.80	158.00	35.00	1.00
131	.03	7.30	6.10	.70	9.40	403.00	40.00	1.00
132	.02	6.30	4.50	1.00	11.60	235.00	40.00	1.00
133	.01	7.90	5.60	1.50	11.40	321.00	90.00	2.00
134	.02	4.20	2.20	1.30	11.80	294.00	45.00	1.00
135	.01	9.20	6.20	1.60	12.10	366.00	55.00	2.00
137	.01	9.60	7.60	1.00	13.00	251.00	100.00	2.00
136	.03	8.60	6.60	1.10	14.10	207.00	20.00	.00
138	.01	6.80	4.20	2.00	10.60	347.00	30.00	.00
139	.02	4.20	4.30	.90	12.10	245.00	40.00	1.00
140	.04	6.90	4.10	2.10	13.10	308.00	45.00	1.00
141	.01	8.70	6.60	1.50	10.50	300.00	110.00	2.00
142	.02	9.10	7.10	1.10	11.10	457.00	80.00	2.00
143	.01	6.60	4.40	1.30	8.00	305.00	25.00	.00
144	.03	8.00	4.80	2.10	12.80	330.00	55.00	2.00
145	.02	7.70	5.00	2.00	13.50	335.00	35.00	.00
146	.02	7.60	5.30	1.40	11.70	267.00	50.00	2.00
147	.02	7.10	4.60	1.20	11.90	319.00	45.30	1.00

Size of tumour 10-35mm = 0, 35-50mm = 1, 50-130mm= 2; Differentiation well= 0, moderate= 1, poor= 2; Lymph node status (negative/ 1-3/ 4+); Lymphatic invasion (negative= 0, positive= 1); Venous invasion (negative= 0, positive= 1).

Patient No	Differentiation Well =0,mod =1, poor =2	Lymph node metastasis	Lymphatic invasion	Venous Invasion
1	1.00	0/0	.00	.00
2	1.00	1//16	.00	.00
3	1.00	0/23	.00	.00
4	1.00	0/21	1.00	1.00
5	.00	0/1	.00	1.00
6	.00	1//9	.00	.00
7	1.00	1//7	.00	.00
8	1.00	0/6	.00	.00
9	1.00	0/24	.00	.00
10	2.00	3//17	1.00	1.00
11	1.00	2//5	1.00	.00
12	1.00	0/14	.00	.00
13	1.00	0/0	.00	.00
14	1.00	0//19	.00	.00
15	1.00	0/16	.00	.00
16	1.00	0/7	.00	.00
17	1.00	0/13	.00	.00
18	1.00	7//11	1.00	1.00
19	1.00	5//5	.00	.00
21	1.00	0/5	.00	.00
20	.00	0/15	.00	.00
22	1.00	0/11	.00	.00
23	1.00	0/10	.00	.00
24	1.00	2//6	.00	.00
25	1.00	0/8	.00	.00
26	1.00	0/10	1.00	1.00
27	1.00	4//7	1.00	1.00
28	1.00	1//18	.00	.00
29	1.00	1//14	.00	.00
30	1.00	1//15	.00	.00

Patient No	Differentiation Well =0,mod =1, poor =2	Lymph node metastasis	Lymphatic invasion	Venous Invasion
31	1.00	0/20	.00	.00
32	1.00	7//34	1.00	1.00
33	1.00	2//17	.00	.00
34	1.00	2//9	.00	.00
35	1.00	5//10	.00	.00
36	1.00	2//5	1.00	1.00
37	1.00	0/22	.00	.00
38	1.00	1//14	1.00	1.00
39	1.00	1//10	.00	.00
40	1.00	0/4	.00	.00
41	2.00	3//13	1.00	1.00
42	1.00	2//34	.00	.00
43	1.00	2//7	.00	1.00
44	1.00	0/20	.00	.00
45	1.00	0/1	.00	.00
46	1.00	0/15	.00	.00
47	1.00	0/4	.00	.00
48	1.00	0/17	.00	.00
49	1.00	1//14	.00	.00
50	2.00	0/13	.00	.00
51	.00	4//9	.00	.00
52	1.00	0/17	.00	1.00
53	1.00	1//9	1.00	1.00
54	.00	0/22	.00	.00
55	1.00	10//18	.00	.00
56	.00	0/24	.00	.00
57	.00	0/8	.00	.00
58	.00	2//10	.00	.00
59	1.00	0/15	.00	.00
60	1.00	2//19	.00	.00

Patient No	Differentiation Well =0,mod =1, poor =2	Lymph node metastasis	Lymphatic invasion	Venous Invasion
61	1.00	2//7	.00	.00
62	1.00	0/11	.00	.00
63	.00	0/8	.00	.00
64	1.00	0/14	.00	.00
65	.00	0/5	.00	.00
66	2.00	4//14	.00	1.00
67	1.00	2//11	.00	1.00
68	1.00	2//11	.00	.00
69	.00	0/22	.00	1.00
70	2.00	3//12	.00	.00
71	1.00	0/19	.00	.00
72	1.00	0/35	.00	.00
73	1.00	0/27	.00	.00
74	1.00	2//13	.00	.00
75	1.00	0/34	.00	.00
76	1.00	0/24	.00	.00
77	1.00	0/12	.00	.00
78	1.00	7//15	1.00	00
79	1.00	3//8	.00	1.00
80	1.00	2//14	.00	.00
81	1.00	0/8	.00	.00
82	1.00	0/5	.00	.00
83	2.00	0/15	.00	.00
84	.00	0/23	.00	.00
85	1.00	1//23	.00	.00
86	1.00	0/9	.00	.00
87	1.00	0/12	.00	.00
88	1.00	0/9	1.00	1.00
89	1.00	1//9	1.00	.00
90	1.00	0/12	.00	.00

Patient No	Differentiation Well =0,mod =1, poor =2	Lymph node metastasis	Lymphatic invasion	Venous Invasion
91	.00	5//19	1.00	.00
92	1.00	0/10	.00	.00
93	1.00	0/7	.00	1.00
94	1.00	0/12	.00	.00
95	2.00	0/16	.00	.00
96	1.00	0/14	.00	.00
97	1.00	0/10	.00	.00
98	1.00	1//19	.00	1.00
99	1.00	0/13	1.00	1.00
100	1.00	5//18	.00	.00
101	1.00	0/25	1.00	1.00
102	1.00	0/26	.00	.00
103	1.00	6//10	.00	.00
104	1.00	0/17	.00	.00
105	.00	0/20	.00	.00
106	1.00	0/15	.00	.00
107	1.00	6//16	.00	.00
108	1.00	0/9	.00	.00
109	1.00	0/16	.00	.00
110	1.00	1//17	.00	.00
111	2.00	1//8	.00	.00
112	1.00	4//24	1.00	.00
113	1.00	0/30	.00	.00
114	1.00	2//14	.00	.00
115	2.00	15/29 C2	1.00	1.00
116	1.00	0/29	.00	.00
117	1.00	0/14	.00	.00
118	1.00	0/7	.00	.00
119	1.00	0/21	.00	1.00
120	1.00	0/12	.00	.00

Patient No	Differentiation Well =0,mod =1, poor =2	Lymph node metastasis	Lymphatic invasion	Venous Invasion
121	1.00	0/23	.00	.00
122	1.00	1//17	.00	1.00
123	2.00	3//25	.00	.00
124	2.00	0/14	1.00	.00
125	1.00	0/8	.00	.00
126	1.00	0/20	.00	.00
127	1.00	0/11	.00	1.00
128	.00	1//11	00	00
129	1.00	0/11	.00	.00
130	1.00	0/10	.00	.00
131	1.00	0/7	.00	.00
132	1.00	1//21	.00	.00
133	.00	0/13	.00	.00
134	1.00	0/35	.00	.00
135	1.00	0/14	.00	.00
137	2.00	3//15	1.00	.00
136	1.00	0/24	.00	.00
138	.00	0/19	.00	.00
139	1.00	6//12	.00	1.00
140	1.00	0/19	.00	.00
141	1.00	2//20	.00	.00
142	1.00	0/21	.00	.00
143	2.00	0/14	.00	.00
144	1.00	0/13	.00	.00
145	1.00	1//9	1.00	1.00
146	1.00	0/18	.00	.00
147	.00	0/22	.00	.00