GLYCAN, TUMOUR SUPPRESSOR GENE AND ONCOGENE PRODUCT EXPRESSION IN GASTRIC CARCINOMATA

A Thesis Submitted To The
Victoria University of Manchester
For The Degree of Doctor of Philosophy
In The Faculty of Medicine

1998

CHONG JIANG

Division of Molecular Pathology
Department Of Pathological Sciences

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ABSTRACT

The scale and potential function of alterations in glycans are yet to be established in gastric carcinogenesis and little is known about the relationship between the alterations in glycans and the expressions of oncogenes and tumour suppressor genes in gastric carcinogenesis and tumour progression.

In this study, a large panel of lectins was used to explore changes in gastric glycans. The abnormal expressions of selected oncogene and tumour suppressor gene products were studied in relation to gastric neoplasia and the relationship between their abnormal expression and alteration in glycans was explored.

In normal gastric tissues, four patterns of lectin staining were observed. First, there were several lectins that selectively stained the mucinous epithelial cells (surface and pit cells). This group included BSA1-B_4, VVA, SBA and CTA. It implies that some saccharide sequences are mainly expressed in the glycans of the mature epithelium.

Second, there were several lectins that showed selective staining mainly on neck cells and gland cells and most of them showed staining predominantly on chief cells rather than on parietal cells. This included GNA, NPA, UEA-1, LTA, PTA, HPA, BPA and BSA-II. This suggests that certain sugar residues are selectively expressed in primitive epithelial cells and cells differentiating towards gland cells.

Third, there were several lectins that stained all mucosal cells uniformly in intensity. This group included ECA, MPA, STA, DSA and BSA-II, implying that certain glycans were constantly expressed during the process of cell differentiation.

Fourth, there were a number of lectins that only stained mucosal epithelial cells, but none of the stromal tissues. They could, potentially, serve as mucosal epithelial cell markers and help in determining whether anaplastic tumour cells were from epithelial origin. These lectins included GNA, NPA, UEA-1, LTA, PNA, BSA1-B_4, MAA, VVA, WFA, SBA, DBA, CTA and BSA-II.

The staining patterns of gastric carcinoma were also of four types. First, diminished staining in tumour tissues, over normal counterparts, was shown by lectins BSA1-B_4, SBA and VVA. They selectively stained normal surface and pit epithelium and they showed staining in few tumour cells in most types of gastric carcinomata. This implies that the majority of tumour cells tend towards lessened differentiation (i.e. increased anaplasia) relative to their normal counterparts and have lost, at least in part, the characteristics of normal mature cells, leading to the alteration of those glycans which are normally typical of mature epithelium.

Second, the elevated staining of glycans was observed in tumour tissues, as shown by MAA. MAA, which rarely stained normal gastric mucosal epithelium, showed increased staining in tumour tissues. It suggests that the α2,3 sialylation of glycans is enhanced during gastric carcinogenesis and is retained during tumour progression. Hence, MAA may be useful indicators in gastric oncogenesis.

Third, some lectins (ECA, MPA, STA and DSA, BSA-II) retained their staining intensity in tumour tissues at the same level as in the normal controls, suggesting that the syntheses of certain glycans are not altered during tumourigenesis and progression. This also implies that the alteration of glycosylation in these tumours is selective and that there is not a global altercation of glycans during gastric carcinogenesis and progression.

It was also noted that some lectins showed more complex variations in their staining intensity, increased in some tumours and decreased in others, such as PNA, PSA, LCA and LTA. This suggests that tumour cells lose the capacity for synthesising these glycans at some stages of tumour progression and regain that ability at later stages, or vice versa.

Studies on the expression of oncogene and tumour suppressor gene products showed that Bcl-2, *ras,* Rb and p53 proteins were frequently expressed in gastric carcinoma. This supports the proposition that the expression of these oncogene and tumour suppressor gene products is associated with gastric carcinogenesis and later progression. Mechanisms for their abnormal expression were proposed.

Changes in certain glycans were associated with the expressions of oncogenes and

tumour suppressor genes. It was notable that the alteration in glycans detected by PNA, MAA, SBA and HPA showed relations to changes in Bcl-2, *ras* and p53 proteins, and PCNA. This implies that some changes in glycans may be general responses to cell proliferation. Since MAA rarely stained normal gastric mucosa and showed increased staining in most tumour tissues, suggesting that there may be a new, novel, glycan synthesised during gastric carcinogenesis and tumour progression.

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Education:

The author received his first degree in the Norman Bethune University of Medical Sciences, Changchun, China in 1984 in the field of medicine and obtained his second degree, M.Sc., in the Department of Pathology in the same university. Since then, the author, as a researcher and lecturer, has been actively engaged in several research projects in the field of Virology, Immunopathology, Oncology and Toxicology.

TO: MY PARENTS, WIFE AND CHILDREN

ACKNOWLEDGEMENTS

I am very greatly indebted to Dr RW Stoddart for his full and strong support, advice, instructions and all the help throughout three years. In respect of the research work, without him, the production of this dissertation would not have been easier and possible.

I am also very grateful to Professor J McClure for kindly being my supervisor and having provided such an opportunity for me to be skilfully trained in his department.

I would also like to express my sincere thanks to all the members of this department for their technical assistance and kindness for providing a comfortable study environment.

CHAPTER 1 INTRODUCTION

Gastric carcinoma is a common abnormality in the stomach. Because of its high incidence and high mortality rate which are the greatest among all diseases in the stomach and even of the digestive tract as a whole, it attracted a considerable body of research. Studies have been performed on the aetiology, occurrence, mechanisms, progression, prevention and treatment of the disease, but efforts are needed to thoroughly understand this disease and to bring it under control.

1.1 BASIC EPIDEMIOLOGY OF GASTRIC CANCER

Gastric carcinoma is a world-wide disease with a very high death rate. Every country in the world has cases of this malignancy, only the relative frequency of cases varies. The incidence rates have dramatically decreased in western countries, such as the United Kingdom and the United States (Whitehead *et al*, 1974; Dupont *et al*, 1978; Preece *et al*, 1986), but elsewhere it is still a common and, in places, a leading cause of all deaths. In China, 56 males and 21 females per 100,000 population suffer from this disease and in Japan, 88 males and 42 females have it. This incidence is the highest in the world (Waterhouse *et al*, 1982).

In an investigation recently published (Parkin *et al*, 1993), estimates were made of the incidence of eighteen major cancers in 24 areas of the world and showed that gastric carcinoma ranks second to lung cancer world-wide, but is the dominant neoplastic disease in some countries including Japan and China.

In China, gastric carcinoma occurs in adults of any age, but is mostly found between the ages of 35 and 65 years old and is highest between 45 and 55 years old in males and 40 to 50 years

in females. The areas with highest incidence of gastric carcinoma include Shandong, Fujian and Henan provinces. It is a leading cause of death in China (Wu *et al*, 1987).

1.2 HISTOLOGY OF GASTRIC CARCINOMA

Gastric carcinoma can occur in any part of the stomach, and generally the pyloric region (40%), and the lesser curvature in particular, has the highest incidence, followed by the cardia (12%) and the fundus.

Grossly, gastric carcinoma is categorised into three types. The first is, the 'extophytic', which refers to the protrusion of the tumour and leads to fungating, papillary growths; there may be haemorrhage, necrosis and ulceration on the surface. The second, the 'flat' or 'depressed', tumour grows inside the gastric wall, leading to its thickening. In the 'excavated', necrosis occurs in the tumour tissues, resulting in the formation of crater in the tumour with an uneven bottom. These types can be seen in both early gastric carcinoma (i.e. a tumour that has not extended beyond the mucosal muscle layer) and advanced tumours. Local and distal metastasis of lymph nodes and intravascular dissemination are often seen.

Microscopically, nearly all gastric cancers are adenocarcinomata (95%) and the rest consists of lymphomata, leiomyosarcoma and tumours from other origins, such as neuroendocrine cells (G-cells). Adenocarcinomata are generally composed of one or more of the following four kinds of cells: foveolar, mucopeptic, intestinal and goblet cells (Fiocca *et al*, 1987). The tumour tissues consist of glandular, tubular or papillary structures or appear in cell masses or scatter in the stromal tissues. Both tumour tissues and cells show different degree of atypia with undifferentiation, in terms of irregularity of glandular structures, cell and nuclear sizes and active and abnormal cell divisions.

1.3 CLASSIFICATION OF GASTRIC CARCINOMA

Many systems for the histological classification of gastric carcinoma have been proposed based upon either macroscopic or microscopic criteria or both. The following table is the summary of commonly used classification systems.

Table 1.1 Classifications of Gastric Carcinoma

Stout (Atlas of Tumour Pathology) 1953
Fungating
Penetrating
Spreading
Superficial Spreading
Linitis Plastica
No special type
Lauren 1965
Intestinal
Diffuse
Ming 1977
Expanding
Infiltrating
World Health Organisation 1977
Papillary
Tubular
Mucinous
Signet Ring
Japanese Society for Gastric Cancer 1981
Papillary
Tubular
Poorly Differentiated
Mucinous
Signet Ring

Borromann introduced the earliest classification system in 1926. Based on macroscopic morphology, he grouped gastric carcinoma into four types: I, polypoid, with polyp-like appearance; II, ulcerating, with the formation of an ulcer in the tumour; III, combined ulcerating and polypoid, with both of the features above and IV, infiltrating, invading deep layer(s) of gastric wall. In 1941, Schindler proposed a similar classification system. He also divided gastric carcinoma also into four types according to observations made with the gastroscope (invented by him). These were: polypoid, the same as Borromann's type I, plateau-like, with the protrusion of tumour over the gastric mucosal surface, ulcerating, the same as Borromann's type III, the fourth, flat type.

Soon afterwards, several more systems of classification were proposed by other investigators, in attempts to reflect and employ all the pathological changes in the tumour tissues. For example, Stout (1953) combined gross and microscopic morphologies of gastric carcinoma in his classification and emphasised gross side of tumour growth. The other systems categorised gastric carcinoma primarily by its microscopic appearance (Ming *et al*, 1977; Oota and Sobin, 1977; Kaibara *et al*, 1987; Kato *et al*, 1981).

The World Health Organisation (WHO) now classifies gastric adenocarcinoma into four types: Papillary, Tubular, Mucinous and Signet ring cell carcinomas (Oota and Sobin, 1977). The undifferentiated carcinoma is classified separately from adenocarcinoma. WHO also grades adenocarcinomata as of well, moderately or poorly differentiated types. This classification has proved to be widely useful for routine diagnostic purposes.

Ming's classification (Ming, 1977) is based on a combination of pathological changes with the

biological behaviour of gastric carcinoma. This classification takes a number of factors such as tumour location, gross tumour form and cellular features, into consideration, and divides gastric carcinomata into two types: Expanding and Infiltrating. According to his study, 67% of gastric carcinomata could be attributed to the expanding type while the remaining 33% were of the infiltrating type.

The Japanese Society for Gastric Cancer (1981) has proposed a very similar classification system to that of WHO, in which the only difference is that they listed the poorly differentiated type separately.

The most widely accepted classification in the West is still that proposed by Lauren in 1965, which divides gastric carcinoma into two categories: Intestinal and Diffuse (Lauren, 1965). The intestinal type is further subtyped according to the pattern of differentiation of the tumour. The signet ring type of gastric carcinoma was considered to be a special variant within the 'diffuse' category.

Intestinal-type carcinomata are usually thought to arise from the metaplastic epithelium and are commonly well differentiated, consisting of papillary, tubular or glandular structures. With increasing anaplasia, the tubular and glandular components become fewer and even disappear, leading to tumours which are solid cell masses of poorly differentiated or undifferentiated types. In high grade neoplasms, tumour cells are much like the resorptive intestinal epithelium and are characterised by columnar shape, large size, a clear cellular border and prominent basilar nuclei with macronucleoli. Cytological atypia is paralleled by structural atypia; i.e. the abnormalities of the cells probably lead to the anomalies of larger scale structures in the tumour.

Diffuse-type gastric adenocarcinoma is always poorly differentiated and lacks glands or similar structures. It is usually composed of loosely scattered cells or cord-like forms with the cells being pleomorphic (i.e. of various sizes, shapes and forms). A form of carcinoma composed predominantly of round cells, with clear cytoplasm or intraplasmic vacuoles and nuclei displaced to one side, is termed 'signet ring cell' carcinoma and is regarded as a subset of the diffuse type. Some carcinomata, indeed, show mixtures of the two histological types above.

1.4 GASTRIC 'PRECANCEROUS' LESIONS

Gastric cancer usually arises on a background of some pre-cancerous lesion such as chronic atrophic gastritis, chronic peptic ulcer, adenoma or dysplasia (Morson *et al*, 1980; Ida and Kusama, 1982; Segura and Montero, 1983; Ming *et al*, 1984; Snover *et al*, 1985).

1.4.1 Peptic ulcer and adenoma

From a general point of view, gastric peptic ulcer has a relatively low potential for malignant transformation, with an incidence of possibly no more than 1% (Morson *et al*, 1980). However, studies on adenoma have claimed that 25-75% of cases are accompanied by malignancy (Snover, 1985).

1.4.2 Chronic atrophic gastritis

It is generally acknowledged that emphasis should be put on chronic atrophic gastritis, because of its close relationship with gastric cancer in terms of their common geographic distribution, anatomical coexistence and the similarity of their associated epithelial changes, including intestinal metaplasia. This means that in those areas where a high incidence of

chronic gastritis exists there is also an excess incidence of gastric carcinoma.

Histopathological evidence supports that of epidemiology, in that most cancers of the stomach have accompanying chronic atrophic gastritis and that chronic atrophic gastritis usually has associated intestinal metaplasia to a variable extent. Intestinal metaplasia of the gastric mucosa means that the cells of the gastric mucosa have come to resemble those of the small intestine or colon, with appearance of various numbers of goblet cells. Jass *et al* categorises this metaplasia as of two histological types: 'complete' and 'incomplete'(Jass *et al*, 1980).

In the 'complete' type of intestinal metaplasia (type I), the crypts are straight and regular with two kinds of cells co-existing: the first of these is goblet cells, which secrete sialomucins with a high proportion of N-acylated derivatives. These mucins are stained dark blue with PAS and Alcian blue. Cells of the second type are 'absorptive cells', which are non-secretory and have well developed brush borders. The 'incomplete' type of intestinal metaplasia (type II) has elongated or torturous crypts with mild dysformation of structure. The absorptive epithelium is characteristically sparse or absent, instead, columnar mucous cells are present in various stages of differentiation. They secrete neutral and/or small amounts of sialomucins. The goblet cells occasionally produce sulphomucins. This type of metaplasia is the least common, but 90% of patients with it have gastric carcinoma (Silvia and Filipe, 1986). Other authors have also suggested that severe intestinal metaplasia carries an extremely high risk of progression to a carcinoma (Silvia and Filipe, 1987; Ida and Kusama, 1982; Segura and Montero, 1983). Intestinal metaplasia most often progresses to dysplasia, which has been considered to be of great importance in predicting the risk of development of carcinoma of the stomach (Ming *et al*, 1984).

1.4.3 Gastric dysplasia

Dysplasia is regarded as a change within the gastric mucosa (Morson *et al*, 1980). It is distinguished from both hyperplasia and carcinoma. Hyperplasia is a simple hyperproliferation of gastric tissues without any obvious cellular atypia and structural distortion.

Gastric dysplasia is associated with many diseases of the stomach, but it is most often found in association with chronic atrophic gastritis and overt malignancy. Because of the high frequency of its anatomical association with gastric malignancy, this gastropathy has attracted heightened interest wherever it occurs in the absence of clear neoplasia.

Gastric dysplasia is classified into three grades according to the degree of cellular atypia, abnormal cellular maturation and disarranged mucosal structure (Grundman and Schlake, 1979). They are as follows.

1) Mild dysplasia. This is characterised by atypical foveolar epithelium emerging in the upper half of an elevated or eroded lesion. The nuclei are slender and hyperchromatic and crowded, but still stay at the base of epithelium. Granular cysts are often present in the deeper layer of the mucosa. There may be intestinal metaplasia present.

2) Moderate dysplasia. One predominant change in this stage is that nuclei are not only hyperchromatic and crowded, but are also piled up and appear stratified (pseudostratified). Numerous cystically dilated and disarranged glands are seen in the lower part of the lesion, but atypical epithelium does not occupy the full mucosal layer and leaves the base of glands covered with pyloric or pseudopyloric glands.

3) Severe dysplasia The most obvious histology in this is severe cellular atypia. Without this change, a diagnosis of severe dysplasia can not be made, even though other changes appear. In the area of intestinal metaplasia, it is deficient in goblet cells and Paneth cells. Nuclei show a dense and stratified arrangement and some even reach the luminal surface of epithelial cells. It is very similar to pre-invasive carcinoma (carcinoma *in situ*), but, in contrast, the nuclei maintain some degree of uniformity.

Some investigators have used different classifications and have variously either categorised dysplasia into several grades or attributed it to possible carcinoma (Ming *et al*, 1984). In other words, dysplasia can be seen as a very severe precancerous lesion with high risk of progression into carcinoma or as a grossly disordered epithelium already in coexistence with gastric carcinoma.

Hence, gastric dysplasia is an indicative link between benign lesions such as gastric ulcer, chronic atrophic gastritis, etc., and carcinoma *in situ* (intra-epithelial carcinoma) as well as invasive carcinoma, and has a great tendency towards eventual, overt malignancy. It denotes excessively abnormal proliferation of gastric epithelium with cellular atypia, abnormal maturation and disordered mucosal structures. It was found that the common type of gastric carcinoma that developed from dysplasia was of the intestinal form (Nishizawa and Okada, 1981, Tosi *et al*, 1987). In a ten-year follow-up study made by Bearzi *et al*, 125 cases of diagnosed dysplasia patients were followed up (Bearzi *et al*, 1984). Among them 81 were low grade and 44 high grade. During a ten-year period, 23 cases (14 low grade and 9 high grade) progressed into carcinoma. This study provides further supportive evidence for regarding dysplasia as a precancerous lesion and a marker for early diagnosis of gastric carcinoma (Tosi *et al*, 1984).

It appears that the following sequence is the evolutionary process whereby normal gastric epithelium converts into malignant epithelium- superficial gastritis, chronic atrophic gastritis, intestinal metaplasia, dysplasia, carcinoma *in situ*, and, finally, invasive carcinoma (Bearzi *et al*, 1984)

A variety of investigations, including a study of dysplasia in experimental carcinogenesis in animals and follow-up studies of patients with biopsy-proven dysplasia (Correa *et al*, 1976; Kunz *et al*, 1979), have shown that mild and moderate dysplasias are probably not clinically significant and are largely reactive in nature, since dysplasia of these types showed either subsequent regression or no further change. Another study by Oehlert *et al* (1979) also proved that mild or moderate dysplasia either regressed or became stationary. Only severe dysplasia was topographically clearly and closely associated with carcinoma (Kunz *et al*, 1979; Farini *et al*, 1982; Meister *et al*, 1979). Even though there is some disagreement over the malignant potential of dysplasia of different degrees, it is obvious that this abnormality plays some important part in gastric carcinogenesis.

1.5 THE AETIOLOGY OF GASTRIC CARCINOMA

Thus far, the pathogenesis of this cancer, in common with most other tumours, remains largely uncertain. Its aetiology is multifactorial, with no single factor being dominant and readily identifiable. Hence, the aetiology and pathogenesis of carcinoma of the stomach involve consideration of a number of distinct factors.

1.5.1 Hereditary factor

The extent of inheritance of susceptibility to gastric carcinoma is limited, but investigations

suggest that it is not negligible (Woolf *et al*, 1950; Xu *et al*, 1981). Woolf *et al* first reported the family clustering phenomenon in 1950. It was found that in the small and isolated state of San Marino, with an extraordinarily high incidence of stomach cancer, 25% of the patients had direct relatives (brothers, sisters and their offspring) affected by this disease, while in only 5.6% of a control population was this observed. Similar genetic tendencies are indicated by recent findings from other countries. For example, in China, a study in 1981 showed that the risk of incidence of gastric carcinoma was nearly 8 times higher in the blood relatives of victims than in non-relatives (Xu *et al*, 1981). Some further investigations of this inherited trend have provided evidence for its being more marked for the diffuse type of gastric carcinoma than in the intestinal type (Laurence's classification).

Other studies have claimed that this inherited predisposition was more pronounced in female patients rather than males and in patients with blood group A rather than those of groups O, B or AB (Lehtola, 1981; Mecklin *et al*, 1988). These studies imply that genetic factors may be of great importance in the genesis of gastric carcinoma and that they may have some relation either to the carbohydrates expressed on the cells and/or secretions of those who are susceptible, or that genes alternately regulating glycan synthesis and expression are linked to those, influencing susceptibility. It must be emphasised that the mechanisms of this 'family clustering' phenomenon seen with gastric carcinoma are not well understood, but genetic predisposition is usually considered to be its main basis. However, a recent report has revealed that infection with *Helicobacter pylori* could be a possible common feature in the clustering of gastric carcinoma, either because they share exposure to a common infection or because they share the inheritance of glycan antigens that make such an infection more likely (Caeneiro *et al*, 1993).

1.5.2 Environmental factors

Other factors, which have known potential relationships to gastric carcinoma, include exposure to certain environmental carcinogens such as nitrosamines and hydrocarbons (Armijo and Coulson, 1975; Ames, 1982). Experimental carcinogens, such as N-methyl-N-nitro-N-nitrosoguanidine (MNNG) have been reported to act as gastric carcinogens (Tsiftsis *et al*, 1980). Dietary habits, lifestyle, occupations and ionising radiation have also been cited as influences (Correa *et al*, 1983; Kohn and Fry, 1984).

Shandong Province in China, which has one of the world's highest rates of gastric cancer, has yielded clues to the environmental determinants of this tumour (You *et al*, 1991). Studies have revealed increased risks associated with consumption of sour pancakes, a fermented staple unique to the area, which contains volatile N-nitrosamines. Within the population consuming this diet, analysis showed high levels of N-nitrosoproline and of N-nitroso-2-methylthiazolidine 4-carboxylic acid in the gastric juice of persons with gastric dysplasia. Similar findings were observed in Fujian province, another area with a high incidence of gastric carcinoma (Chen *et al*, 1992). The tumour has been associated with the consumption of certain salted, fermented, fish products, such as fish sauce. It was found that the levels of N-nitroso compounds and genotoxins were very high.

Radiation-associated gastric cancer has been reported in Japanese exposed to radiation from the atomic bombs during the Second World War and many cases of gastric cancer were found in the survivors of the explosion (Wakabayashi *et al*, 1983). It has also been reported in patients exposed to radiotherapy (Brumaback *et al*, 1984).

1.5.3 Infections

Recent studies have provided evidence suggestive of a correlation between viral or bacterial infections and gastric carcinogenesis.

1.5.3.1 Viruses related to gastric cancer

There are six groups of viruses with DNA genomes involved in human oncogenesis, which include the hepatitis B viruses, simian virus 40 (SV40) and polyomavirus, papillomaviruses, adenoviruses, herpesviruses and poxviruses. Only one viral group with RNA genomes, the retroviruses has been so far implicated. Viruses that appear to have more distinct involvement in gastric cancer are the Herpesviruses, especially type 4, i.e. Epstein-Barr virus (EBV) (Harn *et al*, 1995).

Attempts have been made to establish a relationship between the detection of the presence of the virus in gastric tissues and gastric carcinogenesis. By using various highly sensitive techniques such as *in situ* hybridisation, PCR and RNA sequencing techniques, it was found that EBV occurs in gastric carcinoma tissues in various proportions of cases ranging from 6% to 27%, depending on the methods of detection used (Harn *et al*, 1995; Mori *et al*, 1994; Imai *et al*, 1994; Fukayama *et al*, 1994). The virus has rarely been found in normal, inflammatory, metaplastic or dysplastic gastric tissues. Interestingly, most investigators have emphasised that EBV is detectable predominantly in those undifferentiated tumours with prominent lymphoid infiltration of the stroma (Mori *et al*, 1994; Imai *et al*, 1994; Fukayama *et al*, 1994; Shousha *et al*, 1994; Harn *et al*, 1995; Iezzoni *et al*, 1995), although other types of gastric carcinomata can also show EBV expression, albeit less frequently. For example, Imai and colleagues studied 1000 primary gastric carcinomata by PCR and Southern blot. The

results showed that 70 cases (7.0%) contained EBV genomic sequences. The 'positive' tumours comprised 8 of 9 cases (89%) of undifferentiated lymphoepithelioma-like carcinomata, 27 out of 476 (5.7%) poorly differentiated adenocarcinomata, and 35 out of 515 (6.8%) of moderately to well-differentiated adenocarcinomata. This clearly suggests that EBV is particularly associated with lymphoepithelioma-like carcinomata.

Shibata *et al* (1991) examined eight such cases of undifferentiated gastric carcinoma for Epstein-Barr (EBV) viral sequences using PCR and *in situ* hybridisation (ISH) techniques and detected EBV in seven of the eight cases by PCR. ISH was performed in six of these 'positive' cases and showed that EBV genomes were uniformly present in the carcinoma cells and were not present in the reactive lymphoid infiltrate or normal gastric mucosa.

In order to clarify the nature of the association of EBV with those gastric carcinomata which have stroma rich in lymphocytes, Oda *et al* studied 22 cases of undifferentiated carcinomata with 'predominant lymphoid stroma' by PCR and ISH techniques. EBV DNA was detected by PCR in 14/18 cases (77.8%), including lymph node metastases. Infiltrating lymphocytes and normal epithelia adjacent to carcinoma remained EBV-negative. These findings are consistent with the proposition that EBV infection occurs before transformation of epithelial cells and may be related to oncogenesis in EBV-associated gastric carcinoma and they also confirm that the lymphoid infiltrates lack detectable virus and so are not likely to be its source.

Further analysis of the EBV-positive cases implied that tumour cells presented morphology typical of EBV infection (Uemura *et al*, 1994). This possibly suggests that the role of EBV in

gastric cancer tissues might not depend upon viral population, but upon genetic modifications or alteration that it causes by infection. The detailed mechanisms by which gastric carcinomata are affected remain unclear.

1.5.3.2 Bacterial infection

Studies of bacterial factors have mostly focused on the action of *Helicobacter pylori* (H. pylori). *H. pylori* was first named *Campylobacter pyloridis* (Marshall, 1983) and was then reclassified as *Campylobacter pylori* (Marshall and Goodwin, 1983). Since it has some important differences from other species of Campylobacter in that it has four flagella arising from one pole, secretes large amounts of urease, contains different fatty acids and has a distinctive RNA sequence, it was then reclassified as a new genus gaining its current name, *Helicobacter pylori* (Marshall and Goodwin, 1987).

H. pylori mostly occurs in the stomach, but it is also found occasionally in the duodenal bulb. It is rarely found intracellularly, which suggests that it is a non-invasive bacterium. Its prevalence is world-wide and it is probably the commonest human bacterial infection (Goodwin et al, 1986; Newell, 1991; Koman, 1990), although the incidence varies from country to country. It was also found that the prevalence increased with age, being rarely detectable in children, but being present in 50% of 50 years old persons and up to 75% of those aged over 65 (Koman, 1990).

Morphologically the bacterium is a curved, S-shaped, Gram-negative rod, 2.5 μm long and 0.5 μm wide (Dooley and Cohen, 1988). *In vivo* it usually shows a helical form, but it has a rod-like shape on culture *in vitro*.

Although various disease states associated with this infection have been described, the mechanisms of pathogenicity remain unknown. The easiest virulence factors to be identified are those which enable the organism to colonise the hazardous microenvironment of the gastric epithelium, to survive at this site, and to multiply sufficiently for transmission to a new host. The virulence factors identified to date include the bacterial enzymes urease and catalase, flagellae, and lectin-like adhesins. In addition, it is proposed that the organism has evolved mechanisms to avoid the local antibody responses of the host.

Several putative virulence factors that could directly cause gastroduodenal damage have also been identified (Newell, 1991). These include the direct tissue damage by cytotoxins or urease activity and the indirect tissue damage arising from disruption of mucin integrity. Such mechanisms may contribute to peptic ulcer formation; however, the chronic superficial gastritis most frequently associated with this infection is probably caused by immunopathologic events mediated by the host in response to the continued antigen load on the gastric mucosa.

The most important known feature of *H. pylori* is its secretion of a urease of high specific activity. This enzyme has been structurally and functionally studied in detail (Mobley *et al*, 1991). That it undoubtedly plays a critical role in the pathogeneses, consequent upon *H. pylori* infection, has been shown by a variety of studies (Newell, 1991; Moblley *et al*, 1991; Tytgat *et al*, 1991). This enzyme may well protect the bacterium from the acid of the stomach contents by catalysing the breakdown of urea, which releases ammonium ion to produce a local alkaline environment favourable for the survival of the bacterium.

Much progress has been made with regard to the molecular biology of urease (urea amidohydrolase). This high molecular weight protein (estimated by several investigators to be 300-520 kDa) has been purified (Mobley *et al*, 1991), revealing two distinct subunits of 29.5 kDa and 66 kDa, unlike all other microbial ureases. However, large parts of its amino acid sequences are, nevertheless, well conserved when compared with other bacterial ureases and that of the jack bean, *Canavalia ensiformis*. Furthermore, genes encoding urease of *H. pylori* have been cloned, sequenced, and amplified by the polymerase chain reaction (Courcoux *et al*, 1990; Foxall *et al*, 1990).

Urease catalyses the hydrolysis of urea to yield ammonia and carbon dioxide. Research on this enzyme has gained momentum since the discovery of *H. pylori* as a causative agent of human gastritis. The remarkably high urease activity of each organism has served as the basis of diagnostic tests for its presence by the urease biopsy test and urea breath test (Mobley *et al*, 1991).

Ammonia generated by hydrolysis of urea may also produce severe cytotoxic effects within gastric epithelium by direct injury or by metabolic mechanisms, since the ammonia is known to interfere with the tricarboxylic acid cycle and leads to the dysregulation of gastric cell metabolism. The enzyme also elicits a strong immune response during acute infection, suggesting that this abundant antigen is readily available to the immune system. Furthermore, it may combine with other substances to produce cytotoxic products harmful to the mucous cells (Mobley *et al*, 1991).

In addition to urease, *H. pylori* produces catalase, which prevents the bactericidal effects of neutrophils by degrading peroxide. *H. pylori* also releases protease, lipase, and phospholipase,

which may destroy the integrity of the mucus. All these factors may contribute to gastric pathology. It was also revealed that various inflammatory reactions induced by *H. pylori* infection and its products, including the immune responses, might have roles in the gastropathies (Newell, 1991).

Close relationships have been shown between *H. pylori* infection and several gastroduodenal diseases, including peptic ulcer of duodenum, duodenitis and chronic gastritis (Cramino acidnen *et al*, 1992; Sipponen *et al*, 1992; Farinati *et al*, 1993; Veldhuyzen *et al*, 1994; Kuipers *et al*, 1995) There were also findings suggestive of its association with gastric carcinoma (Loffeld *et al*, 1990; Parsonnet *et al*, 1991; Burruk *et al*, 1993; Cramino acidnen *et al*, 1994; Hansson *et al*, 1995).

There is ample experimental and clinical evidence that *H. pylori* is the cause of chronic type B gastritis (atrophic chronic gastritis). Research has revealed that the bacterium is present in almost all cases of type B gastritis and that antibodies direct against *H. pylori* are serologically detectable. The eradication of the bacterium dramatically improves the efficacy of clinical treatment for most cases of type B gastritis (Rauws *et al*, 1988). Since, in this type of gastritis, gastric metaplasia and even dysplasia usually occur and both are closely related to gastric carcinogenesis, *H. pylori* has been widely conjectured to be an aetiological or pathogenic agent in gastric neoplasia.

Direct evidence for the role of *H. pylori* in the progression to gastric cancer has recently become abundant. Gastric cancer patients showed serological evidence of reaction to *H. pylori* antigens (Hansson *et al*, 1995; Lee *et al*, 1995). Anti-*H. pylori* antibody titres in the sera of stomach cancer patients were significantly higher than in the controls. Further analysis

suggested that *H. pylori* may have a close relation with gastric lymphoma and the eradication of the bacterium can lead to the regression of some tumours, at least in their early stages. Because of its increasing importance in gastric carcinogenesis, the World Health Organisation and the International Agency for Research on Cancer has announced that *H. pylori* is now classified as a definite carcinogen (Eidt and Stolte, 1995).

The mechanisms by which *H. pylori* is involved in gastric carcinogenesis are not well understood. Some have postulated that it acts in causing atrophic chronic gastritis, the accompanying lesions of which, metaplasia and dysplasia, are commonly part of the background of gastric malignancies. Since these lesions are not always present in every patient, it is also supposed that there are other possible ways in which *H. pylori* plays a role in tumourigenesis. Some investigators have raised the possibility that the oxygen radicals originating from the white blood cells as a result of the *H. pylori* infection may induce mutations with carcinogenic potential in the gastric epithelium (Correa, 1995).

However, there has been disagreement about the role of *H. pylori* in gastric carcinogenesis and indeed whether it is involved at all (Clarkson and West, 1993; Prabhu *et al*, 1995). Prabhu *et al* (1995) compared the incidence of *H. pylori* in different gastric tissues and found there was no significant difference among them. This led them to suggest that the role of *H. pylori* infection in gastric tumourigenesis was in doubt. Others have since also obtained similar evidence either supporting the doubt of Prabhu or suggesting that *H. pylori* is, at least, not a direct causal agent in the occurrence of gastric carcinoma (Clarkson and West, 1993; Tanakashi *et al*, 1993).

Nevertheless, the importance of the correlation between *H. pylori* and gastric carcinomas is

gradually becoming established and current evidence suggests that it may correlate best with specific subtypes of gastric carcinoma, especially the intestinal type (Parsonnet *et al*, 1991; Correa, 1995). It has been also proposed that *H. pylori* infection may contribute to the apparent, rather weak, familial inheritance of this cancer (Carneiro *et al*, 1993; Zhao *et al*, 1994).

In conclusion, even though gastric carcinogenesis is multifactorial, no single factor has been shown to be the direct cause of carcinoma of the stomach. Each factor may play some part in the occurrence or progression of this disease, either individually or co-operatively, and in most cases, the malignant neoplasm originates from, or occurs in conjunction with, some antecedent gastric abnormalities the so-called 'precancerous lesions'.

1.6 GASTRIC MUCOSA AND MUCINS

The stomach is the main route by which an individual ingests every nutrient. Accordingly, it is exposed to a wide range of pathogens, toxins and carcinogens, and this is reflected in the diversity of its pathology.

The stomach contains a hostile and highly acidic environment unique in the human body. This acidic environment plays a very important role in protecting the human body by hydrolysing organisms and other potentially harmful substances ingested. The acid in the stomach is separated from mucosa by mucins secreted from surface epithelia. The integrity of the mucin layer is vital for its normal function and the changes of the properties of the mucins can be both extrinsic from the substances ingested and intrinsic from the abnormalities of the mucosa.

1.6.1 The gastric mucosa

Gastric mucosa is conventionally divided into two layers, superficial and deeper, and three histological types, the cardiac, the body or fundic, and the antral or pyloric mucosae. The superficial layer is a lining of columnar epithelial cells of uniform type, while the deeper layer is composed of histologically different cells forming gastric glands (Junqueira *et al*, 1983, Leeson *et al*, 1985)

In the cardiac area, mucous epithelial cells cover all surface and dip down into the lamina propria to form pits or foveolae. These glands usually show simple or coiled tubular structures, present large lumena and are characterised by short pits and relatively thick, coiled glands. Acid or pepsinogen-secreting cells are rare.

Body or fundic mucosa, in contrast, is more complicated and typically includes the following cell types: 1) the superficial mucinous cells, and, in the deeper part, the mucinous neck cells, which synthesise and secrete mucins; 2) the parietal or oxyntic cells, the first recognised hydrogen ion generator, which produce gastric acid with a pH value of around 2.5; and 3) the chief or zymogen cells, and 4) the endocrine cells, containing several kinds of cells which secrete hormones or hormone-like products.

The pyloric area consists of deep gastric pits, into which open tubular or ramified glands, which are unique to the pylorus and are designated as 'pyloric glands'. The cells forming pyloric glands are similar to those in the cardiac region, which mainly secrete mucins, but some may also synthesise enzymes. However, long pits and short branched glands in the

pyloric area present a reverse reflection of the arrangement in the cardiac region. Cells of a further type, the gastrin (G) cells, are intercalated among mucinous cells. They secrete gastrin, a hormone, which stimulates the secretion of acid in the stomach.

1.6.2 The gastric mucins

Gastric mucins, are large molecules, which have long glycan chains covalently attached to relatively small protein core. They are normally neutral, showing no staining or very slight pink with H&E slides and magenta with PAS. These mucins consist of highly glycosylated proteins with large size and complicated configuration (Fischer *et al*, 1983).

The gastric mucins are synthesised and secreted by surface epithelial cells. They form a mucous layer well above the epithelium and separate the acidic gastric contents from the gastric wall. This mucous layer blocks the permeation of the gastric acid, digestion of enzymes in the gastric liquid and the invasion of most pathogenic organisms. Hence, gastric mucins function as a very important barrier in protecting gastric mucosa from acid erosion, protease digestion and invasion by micro-organisms as well as from mechanical injury.

1.6.2.1 The compositions and synthesis of gastric mucins

These mucins show great complexity in terms of their structures. They are heterogeneous glycoproteins with molecular weights estimated to be in the range of 250,000-1,000,000 daltons. These glycoproteins are composed of 60-80% (w/w) carbohydrate, 20-40% (w/w) protein, and 0.3-0.4% (w/w) covalently bound fatty acid (Slomiany *et al*, 1987; Allen, 1981). Much of the carbohydrate is O-linked through α-N-acetyl-galactosamine to serine and/or threonine and mainly consists of N-acetylglucosamine (NAcGlc), N-acetylgalactosamine (GalNAc), galactose (Gal), fucose (Fuc), and sialic (N-acetylneuraminic) acid (NAcNeu)

(Allen, 1981; Neutra *et al*, 1987). The carbohydrate side chains in gastric mucins are complicated in their structures and usually contain several sugars and branched chains. The proteins are composed of four subunits covalently bound together and form the linking protein. Its core is rich in threonine, serine, glycine, and proline. Lipids, especially phospholipids, can further enhance the protective physical properties of the mucus such as viscosity and retardation of hydrogen ion diffusion. Any change of the components can diminish or abolish the protective function of the mucus.

The beginning of the synthesis of mucins occurs in the rough endoplasmic reticulum (rER) of mucin-producing cells. A polypeptide is made upon polyribosomes adherent to the rER. The precursor peptide was believed to be a 60-kDa protein as shown by Sano *et al* (1994). In their study, an approximately 60-kDa subunit of human gastric mucin precursor protein was detected in the intracellular product of human mucous cells. Under non-reducing conditions, they further detected dimer, trimer, and tetramer mucin precursor proteins (120, 180, 240 kDa), the formation of which was not affected by N-glycosylation, as had been proposed by Dekker *et al* (1990). They then suggested that a 60-kDa subunit of the mucin precursor protein was assembled into mature mucin after oligomerization to tetramers for subsequent modification and secretion.

After the synthesis of the protein, the processing and maturation of gastric mucins are followed by glycosylation and sulphation. The sulphation is achieved by the incorporation of sulphate into the mucin core structure by the action of sulphotransferase. This process has been stressed by several reports as an essential step for the formation of the high molecular weight polymer and the integrity of gastric mucus (Slomiany *et al*, 1992; Liau *et al*, 1992). Glycosylation is brought about by the addition of specific saccharide chain(s) in both the rER

and the Golgi apparatus. It is suggested that O-glycosidically linked oligosaccharide is added in the Golgi apparatus, as are the outer chains of N-glycans, while the cores of N-linked sugar chains are 'pre-fabricated' on dolichyl pyrophosphate in the rough ER (Rio-Matin *et al*, 1993) and can be processed in the *cis*-Golgi apparatus. The further modification of synthesised mucins such as molecular modification or combination with other chemical groups may continue afterwards in the Golgi apparatus (Hughes *et al*, 1983).

1.6.2.2 Mucin genes

Genetic research has so far revealed that there are at least eight mucin genes encoding the polypeptide sequences of mucin core proteins. These genes are named MUC1 to MUC8 according to the order of their discoveries and the number is still increasing (Gendler *et al*, 1990; Gum *et al*, 1989, 1990; Porchet *et al*, 1991; Meezama *et al*, 1994; Toribara *et al*, 1993; Boebek *et al*, 1993). The mucin genes responsible for the soluble gastric mucin have been suggested to be MUC5AC and MUC6 (Porchet *et al*, 1995; Toribara *et al*, 1993).

MUC1 was the first mucin gene to be cloned (Gum *et al*, 1989; Spencer *et al*, 1991). The mucin encoded by MUC1 is a transmembrane protein and is the only membrane protein encoded by the mucin genes found so far. This gene is located on 1q21q25 and has 60 base pair tandem repeat sequences, which accordingly direct the mucin protein synthesis with 20 amino acid (amino acid) repeats of the form: PDTRPAPGSTAPPAHGVTSA. The tandem repeat units are the important parts of mucin protein core and the number of tandem repeat units vary from 20 to 80, which leads the molecular weight of polypeptides ranging from 200-500 kDa. There are two variants of MUC1 found (Aplin and Hay, 1995). One is a short transmembrane form known as MUC1/Y, which lacks the tandem repeats and contains an

extracellular domain of 134 amino acids. The other is a secreted mucin which lacks the transmembrane and cytoplasmic domains, but has full length of variable number tandem repeat (VNTR). Their normal physiological functions are not yet known. It has been found that even though the mucins are altered in tumour states, the core remains the same.

MUC2 and MUC3 have both been cloned from human intestinal cDNA libraries and are located on chromosomes 11q15 and 7q22, respectively (Gum *et al*, 1989, 1990). MUC2 has a 69 base pair repeat which encodes the mucin core protein with a 23 amino acid repeat: PTTTPITTTTTVTPTPTPTGTQT, while MUC3 has a 51 base pair repeat with a 17 amino acid repeat in the core protein: HSTPSFTSSITTTETTS. Both mucins are secretory.

MUC4 and MUC5 (including MUC5AC and MUC5B) have been obtained from the bronchial cDNA libraries and MUC5 has been also isolated from gastric cDNA libraries as has MUC6 (Toribara *et al*, 1993; Boebek *et al*, 1993; Meezama *et al*, 1994). Again, they all are secretory mucin genes. MUC4 is located on chromosome 3q29 with a 48 base pair repeat, which encodes a 16 amino acid repeat unit in its mucin protein core. MUC5 is located on 11q15, in the same region as MUC2 and MUC6, and has the shortest base pair repeat of 24, encoding an 8 amino acid repeat in the resultant core protein. It has been found that MUC2 to MUC6 are all expressed in the gastric mucins and the major secreted species has recently been suggested as MUC5AC and MUC6 (Nordman *et al*, 1995; Toribara *et al*, 1993).

MUC5 was once designated as MUC A, B and C. Later it was found that MUCA and C were part of the same gene and then renamed as MUC5AC, while MUC5B appeared to be a different mucin gene, which was cloned from a tracheobronchial cDNA library. By using

peptide sequencing, the gastric mucins were identified as MUC5AC (Klomp *et al*, 1995).

MUC6 is characterised by an individual repeat unit of 507 base pairs (169 amino acids), the longest tandem repeat yet found among the mucin genes. This gene is localised to chromosome 11p15.4-11p15.5. The translated sequence is rich in threonine, serine, and proline (31, 18, and 15% of residues, respectively) and contains a relatively large amount of histidine (7.1% of residues) and alanine (5.6% of residues). Expression of this gene is highest in the stomach and gall bladder, with weaker expression in the terminal ileum and right colon. It is the third mucin to be localised to the 11p15 region besides MUC2 and MUC5, and implies a clustering of secretory mucin genes in this region.

MUC7 is a relatively new gene which was cloned from salivary gland cDNA library (Bobek *et al*, 1993). Human saliva contains high and low molecular weight mucin glycoproteins (MG1 and MG2, respectively) that are structurally distinct. The isolation and characterisation of overlapping cDNA clones which code for the MG2 protein core were carried out by Bobek and colleagues (Bobek *et al*, 1993). DNA sequencing revealed a translated region of 1131 nucleotides encoding a protein of 377 amino acid residues with a molecular mass of 39 kDa. The first 20 N-terminal residues were very hydrophobic and probably comprise the MG2 leader peptide. No sequence homology with any other human or animal mucins, and no significant homology to any other protein was found. MG2 mRNA is about 2.5 kilobases long, and its expression appears to be species, tissue, and cell-specific. The study also found that this gene is not expressed in gastric mucosa.

MUC8 was first reported by Shankar *et al* in 1994. They claimed that a sequence with a

partial 941 base pair cDNA that encoded a 313-amino-acid polypeptide in the tracheobronchial mucins. Between bases 3-892 imperfect 41-nucleotide tandem repeats were found that encoded a unique polypeptide with two types of consensus repeats, TSCPRPLQEGTRV and TSCPRPLQEGTPGSRAAHALSRRGHRVHELPTSSPGGD-TGF. The overall composition of the deduced amino acid sequence matched that expected for a mucin protein core and is rich in serine, threonine, proline, glycine and alanine (51% of residues). Recently they revised the MUC8 repeat sequence as TSCPRPLQEGTPGS and by using fluorescence *in situ* hybridisation, this mucin gene was localised and assigned to the region 12q24.3 (Shankar *et al*, 1997).

Ho *et al* (1995) investigated the expressions of MUC1-6 in various gastric tissues and found different expression patterns among them. They used expression cloning, by screening a human gastric cDNA expression library with antisera against deglycosylated gastric mucin, followed by RNA analysis and immunohistochemistry to quantify and localise mucin gene expression. Their results revealed two clones containing tandem repeats in various gastric tissues, MUC5 and MUC6. RNA analysis indicated that the gastric epithelium contains high levels of MUC5 and MUC6 messenger RNA with little or no MUC2, MUC3, and MUC4 mRNA.

Immunohistochemical analysis showed that surface mucous cells of the cardia, fundus, and antrum expressed MUC5 peptide. In contrast, MUC6 peptide expression was limited to mucous neck cells of the fundus, antral-type glands of the antrum and cardia, and Brunner's glands of the duodenum. In addition, MUC1 showed a polymorphic pattern in normal tissues as revealed by RNA analysis. In the intestinal metaplastic tissues, immunohistochemical staining of MUC1 was rarely detectable. Antibodies against

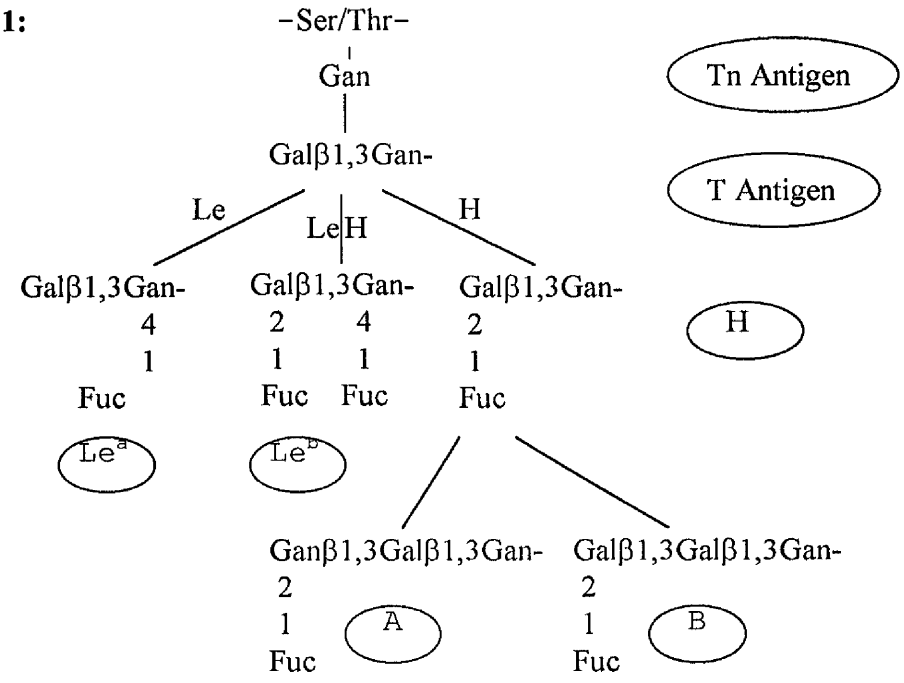
MUC2 and MUC3 showed strong immunoreactivity to goblet cells and columnar cells, respectively. In gastric cancers, the expression and mRNA levels of MUC5 and MUC6 dramatically decreased and only 19 and 57% of the specimens were immunohistochemically detected by anti-MUC5 and MUC6 antibodies. Aberrant expression of MUC2, MUC3 and MUC4 was frequently observed with immunoreactivity of 34% and 45% for MUC2 and MUC3, respectively. They then concluded that MUC5 and MUC6 represented the major secretory mucins in the stomach and were localised to distinct cell types (Ho *et al*, 1995).

1.6.3 Gastric mucins and blood group substances

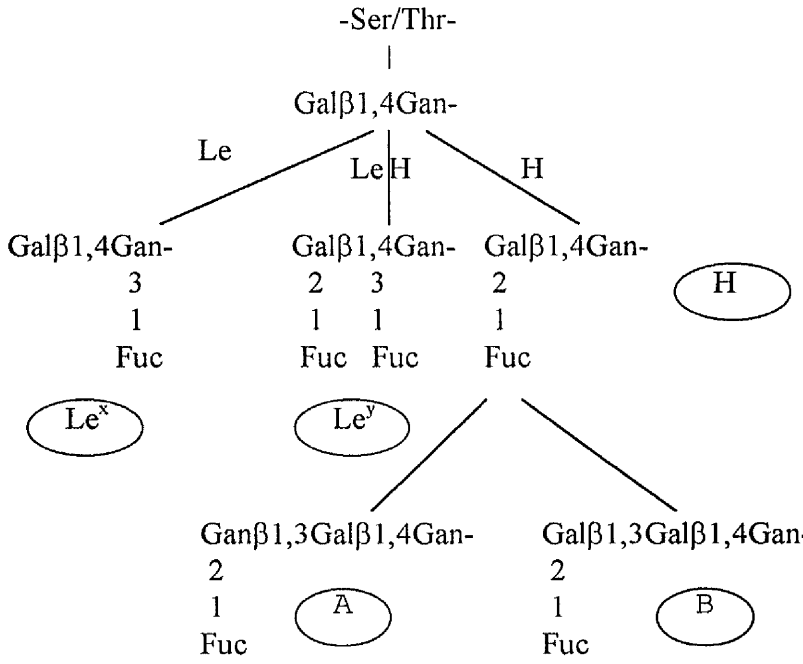
The importance of studying gastric mucins is based not only on the function that they have, but also on the close relations to a wide range of glycoprotein antigens, such as the precursors of blood group antigens, T, Tn, and various blood group antigens derived from them, the ABO and Lewis system, since these antigens often occur in the gastric mucins. ABO and Lewis antigens, together with their precursor T antigens, have been intensively studied in recent years on their roles in the occurrence and development of gastric tumours (Sakamoto *et al*, 1989; Inagaki *et al*, 1990; Torrado *et al*, 1992; Kobayashi *et al*, 1993; Carnerio *et al*, 1994; Dohi *et al*, 1994).

Based on the initial sugar group, N-acetylgalactosamine, attached to the protein backbone and later addition of sugar groups and fucosylation, the above two blood group systems are divided into two families of glycans, type 1 and type 2. The main difference of them starts from the subsequent addition of galactosamine β1,3 N-acetylgalactosamine or galactosamine β1,4 N-acetylgalactosamine. Later, they have a similar fucosylation. Their synthetic processes are illustrated as follows:

Type 1:



Type 2:



Notes: Ser: serine, Thr: threoine, Gan: N-acetylgalactosamine, Gal: Galactose residue, Fuc: fucose residue, H: H antigen, A: A antigen, B: B antigen, Le: Lewis antigen, Le^a-Le^y: Different Lewis antigens.

T and Tn antigens and their sialated forms have been found in a wide variety of tissues and organs, such as salivary gland, gastric tract, pancreas, ovary and in tumour tissues arising from them (Schuessler *et al*, 1991; Inoue *et al*, 1991; Carneiro *et al*, 1994; Fonseca *et al*, 1994).

In the stomach, the patterns of expression of Tn, sialyl-Tn and T antigens were studied in normal gastric mucosa and in the precursor lesions of gastric carcinomata (David *et al*, 1992; Carneiro *et al*, 1994). They found that the expression of the antigens was more clearly related to the cell type and nature of the underlying lesions than to the coexistence of carcinoma. The most distinctive findings were in intestinal metaplasia, dysplasia and hyperplasia. In intestinal metaplasia, Tn was mostly expressed in columnar cells and sialyl-Tn in goblet cells. T was more common in incomplete intestinal metaplasia than in the complete form. High frequencies of sialyl-Tn expression and cell membrane immunoreactivity for T antigen, similar to those found in gastric carcinomata, were observed in adenomatous polyps, hyperplastic polyps, adenomatous dysplasia in the neighbourhood of intestinal carcinomata, and in marked foveolar hyperplasia from the mucosa adjacent to diffuse carcinomata. It was then suggested that adenomatous and hyperplastic lesions shared with gastric carcinomas features of aberrant glycosylation, namely the cell membrane expression of T antigen.

David *et al* (1992) also observed that T antigen appeared to be associated with gastric carcinoma and had an expression pattern distinct from normal gastric tissues: Tn antigen expression was identified in all the normal-looking mucosae; sialosyl-Tn was present in some cases. No T antigen was expressed in normal tissues and sialosyl-T antigen was observed in a few cases of normal tissues. The expression sites of these antigens were limited to the

cytoplasm- mostly in the supranuclear area corresponding to the Golgi apparatus. All the mucosae with intestinal metaplasia showed sialosyl-Tn expression in the goblet cells. In gastric carcinomata, 91.9% of cases expressed Tn antigen, 19.3% expressed sialosyl-Tn antigen, 20.7% expressed T antigen and 19.5% expressed sialyl-T antigen. In contrast to normal mucosa, carcinoma cells expressed simple mucin-type antigens both in the cytoplasm and at the cell membrane. It was observed that most primary carcinomas were concurrently stained for Tn and sialyl-Tn antigens alone (41.1%), or together with T antigen or sialyl-T antigen (28.7%), and that T antigen (and sialyl-T antigen) expression was correlated with the wall infiltration. All of 18 tumours expressing T antigen and 16 out of the 17 tumours expressing sialyl-T antigen had nodal metastases and deep invasion (i.e. wall penetration, lymph node metastasis and venous invasion).

There have been many studies on the roles of blood group antigens, especially Lewis antigens, in the occurrence and differentiation of gastric cancer (Sakamoto *et al*, 1989; Inagaki *et al*, 1990; Murata *et al*, 1992; Torrado *et al*, 1992; Kobayashi, 1993; Dohi *et al*, 1994). Dohi *et al* (1994) recently studied the activity of enzymes for Sialyl Le^a antigen and sialyl Le^x antigen, the cancer-associated carbohydrate antigens, in both benign and malignant gastric tissues. They found that enzyme activity for Sialyl Le^a was detected in most normal or malignant mucosae of gastric tissues, while the enzyme activity for Sialyl Le^x showed low activity in the normal gastric mucosa. However, the Sialyl Le^x transferase was higher in 77% of gastric cancer tissues than in corresponding normal tissues.

Kabayashi *et al* (1993) had earlier reported that the expression of Lewis antigens differed in various normal gastric tissues. $Lewis^b$ antigen was detected by immunohistochemistry

in 95% of normal foveolar epithelial samples, whereas only 10.0% expressed detectable Lewis^a antigen. In contrast, specimens showing intestinal metaplasia had an increased Lewis^a antigen expression and a slightly decreased Lewis^b antigen expression relative to normal controls. Gastric adenomata and adenocarcinomata of intestinal type showed a similar pattern of Lewis^a and Lewis^b expression. Lewis^x and Lewis^y were detected in all normal deep glands, but were not found in the majority of specimens of intestinal metaplasia. Only 20-40% of gastric adenomata and gastric carcinomata expressed Lewis^x and Lewis^y antigens. The results implied that altered expression of Lewis blood group-related antigens may well correlate with pattern and progression in gastric carcinogenesis.

Murata *et al* (1992) observed a high incidence of the inappropriate expression of Lewis type 1 antigens (and the deletion of H and Lewis type 2 antigens (Lewis^x and Lewis^y) both in gastric carcinoma and intestinal metaplasia. The acquisition of CA19-9 (McAb against sialyl Lewis^a) reactivity and the deletion of B antigen were frequently found in intestinal-type cancer and in all types of intestinal metaplasia. The co-deletion of A antigen was found to be limited to the combination of intestinal-type carcinoma with incomplete-type intestinal metaplasia. They concluded that similar changes in carbohydrate antigens were present in early gastric cancer and intestinal metaplasia. These studies also provided evidence that the altered expressions of blood group antigens detected in various gastric tissues could closely relate to gastric carcinogenesis.

1.7 METHODOLOGIES USED IN STUDYING GASTRIC CARCINOMA

Many techniques used for studying normal gastric tissues have also been applied to gastric carcinomata. Many more analytical methods have recently become available and genetic

engineering has brought research on gastric carcinoma to the molecular level.

1.7.1 H&E staining

Haematoxylin and Eosin (H&E) staining is the most widely used routine staining method and the primary diagnostic technique for distinguishing and typing gastric malignancies. Most clinical diagnoses derive from this simple and reliable procedure. It is still widely used in the preliminarily screening of gastric carcinomata.

1.7.2 Histochemistry and immunohistochemistry

Histochemistry and immunohistochemistry cover a wide range of techniques applicable to gastric carcinomata. They are especially useful in the investigation of mucins and the changes that occur in these in malignancy.

Methods such as alcian blue (AB)- periodic acid Schiff's staining and later modifications can basically determine the chemical properties of mucins as being acidic, alkaline or neutral, strongly acid or weakly acid, sulphated or carboxylated, O-acetylated or non O-acetylated, and sialomucin.

Lectin histochemistry is an important advance because this technique can provide more detailed information about glycans of mucins. By comparison between carcinomata and normal tissues, any subtle changes can be useful in defining the role of mucins in gastric carcinogenesis.

Immunohistochemistry has been applied to the study of gastric carcinoma. A number of anti-mucin monoclonal antibodies (McAb) are also commercially available, which dramatically increases the sensitivity and specificity of immunohistochemistry.

Progress on conventional histochemistry and immunohistochemistry has raised studies on mucins to the molecular level, since the change of a single molecule can be detected through an appropriate signal amplification system.

1.7.3 Genetic techniques

Molecular genetics has developed rapidly in recent years and has enabled the genetic basis of biochemical and morphological changes in diseased states to be investigated. By using molecular biology techniques, such as DNA or RNA in situ hybridisation, Northern blot, Southern blot, DNA and RNA sequencing, polymerase chain reaction (PCR), etc., many oncogenes and tumour suppressor genes have been discovered and have been shown to play crucial parts in aetiology and pathogenesis of many malignancies, some of these genes have close relation with the occurrence and progression of gastric carcinomata.

1.8 QUESTIONS TO BE TACKLED IN THIS STUDY

In this study, attempts are made to find answers to the following questions:

1. What are the changes occurring in gastric glycans during gastric carcinogenesis and tumour progression?
2. At what stages of tumour progression do these changes occur?
3. To what extent do changes in glycans reflect the cell growth status, maturation, differentiation and the dynamics of the cell hierarchy?
4. Are there any specific, *novel* saccharides appearing in gastric carcinoma tissues (i.e. that are absent in normal gastric tissues)?
5. Is there any change attributable to purely reactive changes?
6. Is there any change of expression of glycan related to the alteration of oncogenes and tumour suppressor genes?
7. How far does the association of the alteration of glycans and the abnormal expression of oncogene and tumour suppressor gene products reflect the differentiation and progression of gastric carcinoma?

CHAPTER 2 LECTIN HISTOCHEMISTRY IN GASTRIC STUDIES

2.1 HISTORY OF LECTINS

Lectins, are a subset of sugar binding proteins, which are now known to be present in extracts from a wide range of sources, including plants, animals, fungi and bacteria. They were first described in the late nineteenth century by Stillmark, who observed that the extracts of castor bean have haemagglutinating activity (Boyd, 1970). Despite the fact that the species-specificity of lectin haemagglutination was recorded as early as 1908, they failed to attract much interest until 1948 when the human blood group specificity of some lectins was discovered (Boyd, 1949, 1970). At that time the term 'lectin' was coined, from the Latin 'legere', meaning 'to choose' or 'to pick out'.

The term 'lectin' originally meant, variously, 'agglutinin', 'haemagglutinin' or 'phytohaemagglutinin', depending upon whether erythrocytes or other cells were being agglutinated and whether plant, animal or fungal extracts were being used. It was later found that these substances also displayed other properties such as toxicity, mitogenesis and agglutination of malignant cells (Sharon and Lis, 1989). They were present not only in plants, fungi and prokaryotes, but also in animals (Etzler, 1985; Yeaton, 1981a, 1981b). Because of their diverse origins and variation in properties, their definition and nomenclature have been a matter of dispute and they are still, in some ways, controversial.

A widely accepted definition of a lectin, used by the Nomenclature Committee of the International Union of Biochemistry, is that 'a lectin is a sugar binding protein or glycoprotein of non-immune origin which agglutinates cells and/or precipitates complex carbohydrates (glyco-conjugates)'.

Goldstein, *et al* (1980) further suggested that a lectin should have at least two sugar binding sites, agglutinate animal and plant cells and /or precipitate polysaccharides, glycoproteins and glycolipids. In their opinion, the specificity of a lectin is usually determined in terms of monosaccharides or simple oligosaccharides which inhibit the agglutination or precipitation induced by the lectin. They also argued that sugar-specific enzymes, in some cases, act as lectins since those enzymes might have multiple binding

sites and could agglutinate and/or precipitate glycans. They excluded toxins such as ricin D as lectins, even though they are able to combine with sugar, since they have only one sugar- binding site.

However, Kocourek *et al* (1981), expressed their disagreement, in that some saccharide-binding proteins with only one binding site or with toxic or hormone-like activities would be excluded by this standard definition They stressed this was especially true in the case of toxins of *Ricinus communis* (RCA) and *Abrus precatorius* (APA) which are chemically related to lectins. So they suggested that the definition of lectins should include their activities *in vivo* and not only the properties of lectins *in vitro*. In some respects, a new term, such as 'hemilectin', is needed to describe these saccharide-binding proteins.

Generally, lectins are given acronyms which reflect their species of origin and end with 'A' representing 'agglutinin' or 'L' for 'lectin'. For example, the lectin of *Galanthus nivalis* is represented as GNA. *Erythrina cristagalli* agglutinin is ECA. Lectins from the same origin, but with different binding properties, are usually distinguished by the addition of a number or letter as a suffix or prefix. For instance, e-PHA and l-PHA are both from the plant *Phaseolus vulgaris* and are chemically related, but were given different acronyms referring to their individual ability to agglutinate erythrocytes and leukocytes respectively. Examples of the use of a suffix to distinguish very similar lectins are LCA-A and LCA-B of *Lens culinaris* which have effectively identical binding properties. Isolectins which originate from the same species, but have totally different specificity are similarly distinguished by suffixes; for example, BSA1-B_4 and BSA1-A_4 are closely related and interact with α-D-galactopyranoside and 2-deoxy, 2-acetamido-α-D-galactopyranoside termini, respectively, while BSAII is a different protein and bind to 2-deoxy, 2-acetamido-α-D-glucopyranosides.

Lectins are traditionally classified into a small number of specificity groups, namely, mannose, galactose, N-acetyl-glucosamine, N-acetyl-galactosamine, L-fucose and N-acetyl-neuraminic acid. This is largely based upon the monosaccharide which is the most effective inhibitor of the agglutination of erythrocytes or precipitation of carbohydrate polymers by the lectin.

There have been over seven hundred lectin activities found of which a considerable minority have now been purified and characterised, and some of these are commercially available (Sharon and Lis, 1989). For some, the chemistry of their binding is understood in great detail, which has made them important biochemical tools for exploring the components and structures of their specific binding molecules in a variety of situations and models for the interaction of small molecules with proteins (Sharon and Lis, 1972). Table 2.1 summarises some of the many applications which exploit the properties of lectins.

Table 2.1 The main biological activities and applications of lectins

Activities	Application
Blood group specificity	blood typing, and structural studies of components of blood group substances, determination and typing of new blood groups
Mitotic stimulation	exploring chromosome abnormalities and the control of mitosis
Cell agglutination	obtaining information on cell surface structures, their mobility, exposure and modification on transformation
Toxicity to animals	nutritional research, food processing, various forms of dietary sensitivity and malabsorption
Precipitation of polysaccharides and glycoproteins	Isolation, purification and structural studies on them
Others	insulin-like effect, mediating target cell-killing, studies of mechanisms of hormone action; tumour treatment

2.2 METHODOLOGIES USED IN LECTIN HISTOCHEMISTRY

Lectins have been extensively used in biochemical analysis (Sharon and Lis, 1972) and the techniques that exploit them (especially biological labelling techniques) have been

greatly developed in recent years. The development and advance of technology have enabled lectins to be used as very sensitive and specific probes for exploring the finer molecular structures of the saccharide sequences attached to glycoproteins.

One of most important applications of lectins is in serving as biochemical probes for investigating the nature of glycans upon or within a cell, and for analysing those changes in saccharides associated with malignancies, since lectins can detect subtle changes of glycans through selectively binding to terminal sugar groups as well as to those internal, hidden in linear or branching arms, which may have altered during carcinogenesis.

In order to make use of lectins, different probing techniques, including direct labelling and indirect labelling, with isotopes, colloidal gold or enzymes have been developed to study components which are specifically stained with lectins and which are readily observed by light-microscopy or electron-microscopy, as stated below.

Direct labelling of lectins with fluorescein and radioactive isotopes have proved to be rapid methods (Cronin *et al,* 1970; Stoddart and Kiernan, 1973; Roth *et al,* 1978). The shortcomings of direct labelling are lack of sensitivity and the need for a complicated fluorescence microscope. Colloidal gold labelling, either direct or indirect, is a relatively new method, but is expensive. It is especially suitable for electron microscopy, because gold has a very high electron density, which facilitates the study of subcellular organelles where glycans are bound and provides information on intracellular sites of glycan synthesis. However, because of the high cost and strict requirements to avoid artifacts, it is unlikely to become a routine technique.

Enzyme (usually peroxidase) labelling is a much easier and more effective, and now widely used technique. It was first used in histochemistry in the late 1960's and in lectin histochemical studies in 1970. At first, concanvalin-A was directly conjugated with horse-radish peroxidase and was the major reagent of that period (Gonatas and Avrameas, 1973; Parmley *et al*, 1974; Kiernan, 1975). Again, this direct labelling technique has penetration problems. Later, several indirect enzyme labelling techniques came into use, which now provide a powerful set of research tools.

Among them, Jones and Stoddart refined the avidin-biotin-peroxidase method, which has proved to be a more rapid, sensitive, versatile and specific (as well as economical) method of lectin staining than the earlier versions. It is now widely used (Jones and Stoddart, 1986).

This detection system, namely the avidin-biotin-peroxidase complex (ABC) system, employs two substances that possess high affinity to each other. Avidin, which is a glycoprotein, binds with strong affinity and specificity with biotin, a vitamin found widely distributed in animal tissues (Green, 1975; Wood and Warke, 1981). It starts from routine dewaxing, rehydration of a section, followed by removal of endogenous peroxidase through exposure to acidified alcoholic hydrogen peroxide. Then biotinylated lectin is added to the section to combine with its target sugar(s). After several washing, avidin-peroxidase complex is added. For disclosure of the peroxidase, 3,3-diaminobenzidine tetrahydrochloride dihydrate (DAB) is oxidised in the presence of hydrogen peroxide to yield a brown colour. This can be immediately observed under normal light microscope or in the electron microscope because it is electron-dense. The preparations are readily made permanent.

Because this system has the advantages of the convenience, simplicity and their capability of providing structural information about glycans by using only one method but as many probes as possible to detect various sugar sequences, lectin histochemical staining techniques are extensively applied in many areas of biological research. They have also been long applied to study neoplasia, and malignancy.

2.3 LECTINS USED IN STUDIES ON GASTRIC TISSUES

It is well established that lectin histochemistry is extremely useful in the gastrointestinal tract and has revealed far more subtle structural variations in epithelial glycans than those which are shown by "conventional" histochemical methods (Fischer *et al*, 1984). It can discriminate individual sugar residues on an oligosaccharide chain. Moreover, the differences between inter- or intra- cellular sugar components in normal and abnormal mucins may be important indices for the diagnosis and differentiation of carcinoma from various pre- or non-neoplastic pathological conditions. In particular, the secretions of

gastric carcinomata consist, in large part, of mucous glycoproteins which differ from those of normal mucosal cell secretions in the proportions of the major oligosaccharides that they contain (Schrager and Oates, 1973, 1978; Filipe, 1979).

Numerous studies have been performed which show that some lectins selectively label gastrointestinal mucus-secreting cells and bind, via specific sugar residues, in specific configuration (Etzler and Branstrator, 1974; Essner and Chreiber, 1978; Watanabe *et al*, 1981; Sato and Spicer, 1980, 1982a, 1982b, Suzuki *et al*, 1982, Peschke *et al*, 1983; Okamoto *et al*, 1988). These studies have also suggested that lectins can be selective markers for particular kinds of cells or tissues: HPA, for example, is an excellent marker for mucous neck cells (Suganuma *et al*, 1985).

Detailed investigations have been made of different gastric mucosae ranging from normal mucosa through metaplastic and dysplastic to neoplastic mucosa, using a limited number of lectins (Bur and Franklin, 1985; Kuhlmann *et al*, 1983; Fisher *et al*, 1983; Marcatney, 1986; Narita and Numao, 1992). Valuable information has been produced of potential utility in diagnosis and prediction of progression in gastric neoplasia.

Ito *et al* (1985) examined normal human gastric mucosal cells by light and electron microscopy, using DBA, RCA-I and ConA as probes. They found that human gastric mucosal cells revealed specific binding patterns for each lectin by light microscopy. Among the lectins tested, DBA, in particular, gave a characteristic pattern. It specifically stained the supranuclear region of surface epithelial cells and the perinuclear region of parietal cells. By electron microscopy, the stacked cisternae and the vesicles of the Golgi apparatus of the surface epithelial cells were seen to be stained with DBA. These results show that the DBA-positive supranuclear region, observed by light microscopy, corresponds to the Golgi apparatus. In the parietal cells, DBA, RCA and ConA bound to the intracellular secretory canaliculi, which are invaginations of the cell membrane running around the nucleus within the cytoplasm. Therefore, the tubular perinuclear positive region observed by light microscopy corresponds to the membranes of the intracellular secretory canaliculi. In addition, ConA stained the endoplasmic reticulum, Golgi apparatus, nuclear envelope, and cell membrane of the parietal cell, which explains the diffuse cytoplasmic staining observed at the light microscopic level with this lectin.

Eight lectins were employed in Bur *et al*'s study (1985), using fluorescence microscopy, on the binding of lectins to paraffin sections of gastric carcinomata of both intestinal and diffusely infiltrating types, and adjacent mucosa. WGA and RCA-I appeared to bind to both mucous and non-mucous glycoproteins and to label tumour cells, benign epithelial cells, and non-epithelial tissues strongly and consistently. PNA, SBA, DBA, BSA, and UEA-1 bound extensively to 'mucosubstances' in vacuoles and apices of benign epithelial cells, but often bound to tumour cells focally and in some cases did not bind at all. From the results they suggested that the decrease in the proportion of tumour cells labelling with lectin, relative to superficial epithelial cells, could be the result either of the 'over-sialylation' of 'mucoproteins' or arise from the loss of glycosyltransferase activity. ConA did not bind to 'mucosubstances' in the vacuoles or apices of benign epithelium, but bound to mucous vacuoles of metaplastic epithelium and to coarse cytoplasmic granules in two of the tumours examined. This suggests that there is an abnormal addition of mannose to mucous glycoprotein or the production of a distinct glycoprotein by some gastric tumours.

Further subcellular distribution of peanut lectin (PNA) binding sites was examined in normal fundic glands from human gastric samples (Malchiodi *et al*, 1985). A positive reaction was observed in the glycocalyx of the secretory canaliculi of parietal cell as well as in the mucous globules of mucous cells and in the luminal cell coat of chief cells. The presence of terminal galactose in the canalicular glycocalyx may be connected with the peculiar function of hydrochloric acid secretion. It was then proposed that PNA might be used as a marker for visualising the secretory canaliculi of parietal cells.

Parietal cells from human gastric mucosa were studied by Kessimian *et al* (1986). They utilised 12 biotinylated lectins for light microscopic and ultrastructural analysis. Parietal cells reacted strongly with BSA1-B_4, DBA, PNA, and SBA (all specific for galactosyl/ 2-deoxy, 2-acetamido-D-galactosyl groups) and weakly with UEA-1 (specific for α-L-fucose). At the light microscopic level a beaded, perinuclear staining pattern was observed which, ultrastructurally, corresponded to an intense staining of intracytoplasmic canaliculi. The membranes of the intracytoplasmic canaliculi were characterised by an

abundance of galactosyl residues, a paucity of fucosyl groups, and a lack of mannosyl and glucosyl residues.

In other studies, the histochemical binding of fluorescein isothiocyanate-conjugated peanut agglutinin (PNA) to paraffin sections of gastric carcinomata and their adjacent mucosa was investigated (Gan, 1990). It was found that PNA could recognise the terminal Gal β1- and D-Gal β1,3 GalNAc α1- sequence. A high rate of positive staining was obtained in cells of malignant tumours, particularly in cases of signet ring cell carcinoma and mucinous adenocarcinoma. Neuraminidase digestion was seen to enhance PNA binding only in well-differentiated adenocarcinomata and the PNA binding glycan (i.e. the so-called receptor) had no specificity for tumour.

Sarker *et al* (1994) found, in their study, that BPA (*Bauhinia purpurea* agglutinin) appeared to bind linearly to the apical cytoplasm of the normal surface columnar epithelium, but more diffuse staining was observed, in addition to the apical binding, in the cells of gastric tumours, including adenomata and carcinomata. The results of the changing staining patterns between normal and tumourous tissues suggest that the glycans (D-Gal β1,3-D-GalNAc α1-) had undergone an alteration during the differentiation of the cells.

Shue *et al* (1993) found that VVA (*Vicia villosa* agglutinin with a specificity for GalNAc α1,3 Gal β1-) showed binding to 89% cases of gastric carcinomata, in contrast to 41.7% cases in the normal counterparts. They also implied, by the same experiment, that PNA (peanut agglutinin with specificity for Gal β1,3 GalNAc α1> Gal β1,4 GlcNAc β1-) showed little difference in binding between normal and neoplastic tissues.

Narita *et al* (1992) used a set of lectins to study various gastric mucosae and found that, in comparison with normal gastric tissues, metaplastic mucosa showed an increase of ConA binding and a decrease of cytoplasmic reaction with WGA (*Wheat germ* agglutinin), PNA, UEA-1 (*Ulex europaeus* agglutinin) and DBA (*Dolichos biflorus* agglutinin). Intestinal-type carcinomata resembled metaplastic surface cells in their binding to ConA, UEA-1 and WGA, while diffuse carcinomata were similar to normal mucosal epithelium in their cytoplasmic binding with WGA and UEA-1.

Other studies showed that glycans binding DBA were abundant in gastric carcinomata and that l-PHA showed a graded increase of staining in the order of mild, moderate and severe dysplasia and carcinoma (Li and Lei, 1993). The distribution of l-PHA 'receptors' was correlated with the histological type and the degree of differentiation. It was found that more l-PHA binding appeared in the tumours that had metastasised to lymph nodes.

Studies on early gastric carcinomata with the lectins ConA, DBA, PHA, PNA, and WGA also suggested that the distribution of stain and the intensity of positive reaction had correlations with the histologic types of gastric carcinomata, their histogenesis and their patterns of cellular differentiation. It implied that lectins could have value in the diagnosis of gastric malignancies (Li, 1992).

As early as in 1982, Sato and colleagues (Sato and Spicer, 1982b) studied the ultrastructural localisation of peanut lectin-binding sites in gastric surface epithelial cells. This study visualised glycoprotein with terminal galactose residues in the apical cytoplasm of the cells and in secreted mucins. It localised such glycoproteins selectively in the 'intermediate Golgi cisternae', situated between the saccules of the maturing face and those of the forming face of the Golgi stacks. Other cytoplasmic organelles, including the forming and stored secretory granules, did not display glycoprotein with terminal galactosyl residues. These results indicated the possible site in the Golgi apparatus where galactose residues were being added to the growing oligosaccharide side-chains of mucus glycoproteins.

Through the study of the ultrastructural distribution of lectin-binding sites on gastric surface epithelial cells, Rio-Martin *et al* found that all types of cells were positively stained with UEA-1 and PNA in the region of the Golgi apparatus and in mucous vacuoles in the cytoplasm (Rio-Martin *et al*, 1993). The *cis* side of Golgi apparatus was labelled by SBA and PNA. SBA also exhibited affinity for rough endoplasmic reticulum. Based on the findings with SBA as well as with other lectins, the authors postulated that the initiation of O-glycosylation was in the Golgi apparatus, but not in rough endoplasmic reticulum, which might be involved in the N-glycosylation. A hypothesis for sugar addition to the carbohydrate side chains of gastric glycans was formulated, in

which the following sequence of steps occurred. First, GalNAc was added in the *cis* side of the Golgi apparatus, then GlcNAc was added at the intermediate face and GalNAc, Gal or α-L-fucose was added last, at the *trans* side of Golgi apparatus.

Earlier work with the lectins MPA (of *Maclura pomifera*, with a specificity for α-Gal and GalNAc residues), DBA, PNA, RCA-I (of *Ricinus communis*, specific to β-Gal) and LFA (of *Limax flavus*, with affinity for α-NeuAc) by using electronic microscopy gave similar findings (Ihida *et al*, 1991). In the surface mucous cells, the nuclear envelope and rough endoplasmic reticulum (rER) and *cis* cisternae of the Golgi stacks were intensely stained with MPA. In the Golgi apparatus, one or two *cis* side cisternae were stained with MPA and DBA, while the intermediate lamellae were intensely labelled with PNA. Cisternae of the *trans* Golgi region were also stained with MPA, RCA I and LFA. Immature mucous granules which are contiguous with the *trans* Golgi lamellae were weakly stained with RCA I, while LFA stained both immature and mature granules. The differences between each lectin's reactivity in the rough endoplasmic reticulum, in each compartment of the Golgi lamellae and in the secretory granules suggest that there are differences of composition and structure between the glycoconjugates in each of these organelles, reflecting the various processes and steps of glycosylation in the mucous cells of the gastric surface.

LFA has recently been proven useful in subtyping mucous neck cells and intermediate cells (Fujita and Kaneko, 1994). It is generally accepted that the mucous neck cells are the precursors of chief cells and that they are converted to chief cells via intermediate cells. It was shown that the intermediate cells were negative for staining with *Limax flavus* lectin (LFA) while mucous neck cells showed positivity. It was then proposed that the mucous neck cells are converted to chief cells via intermediate cells, which show some alterations in their mucus secretion during the process, and LFA may be used as a tool in recognising their difference.

UEA-1 and LTA are specific for fucose-containing glycoconjugates and their binding to normal and neoplastic human gastric mucosa were histochemically studied by Macartney *et al* (1987). Their results were compared with ABO and secretor status and the immunohistochemical demonstration of a Type 1 antigen (Lewis^a) and two Type 2

antigens (Lewis^{x} and Lewis^{H}) using monoclonal antibodies. Binding of UEA-1 and LTA to surface mucous cells in normal gastric mucosa was only seen in blood group secretors, but was independent of ABO status. In gastric carcinomata, lectin binding was reduced. In contrast, LTA staining was associated with both H Type 2 and Le^{a} antigen, but not with secretor status. The results of the study were consistent with the hypothesis that competitive interaction between fucosyl and sialyl transferases occurred in gastric malignancy and leads to the expression of abnormal blood group-related antigens.

In Macartney's other study, four lectins (PNA, SBA, DBA and HPA) were employed on normal human gastric mucosa and gastric carcinoma (Macartney, 1986). Binding of PNA to surface mucous cells or normal gastric mucosa occurred in non-secretors, but not secretors, and was independent of ABO blood group at all sites. PNA binding was unrelated to the immunohistochemical demonstration of Thomsen-Friedenreich (T) antigen. DBA and HPA bound selectively to surface mucous cells in normal gastric mucosa from group A secretors, but binding at other sites was independent of ABO status. SBA staining showed no relationship with blood group or secretor status. In gastric carcinomata, the major finding was that extensive masking of lectin binding sites by sialic acid occurred, which was not seen in normal mucosa. Sialic acid masking was most marked with PNA binding and was least marked with DBA. There was no correlation between lectin binding patterns and the stage or pattern of differentiation of tumours. These results are consistent with studies *in vitro*, which demonstrated increased sialylation of membrane glycoproteins following malignant transformation.

Other gastric cell types have also been explored by lectin histochemistry as well, such as G cells (gastrin-secreting cells) (Hsu and Raine, 1982).

In summary, previous studies have shown that a number of lectins bind to gastric mucosa in various states, from normal to tumourous tissues. Diverse binding sites and patterns of different lectins have been observed with individual characteristics. Lectins have been proven to be very useful in studying gastric mucins by showing normal patterns of staining and altered patterns in various diseased states.

From previous studies, it is evident that the number of lectins used thus far has been very

limited, in relation to their number available, and that some groups of lectins have never been reported as being applied to gastric tissues. It was, therefore, desirable to use a wider and more logically related range of lectins in an attempt to catalogue the patterns of altered glycosylation in the metaplasia-dysplasia-carcinoma sequence in gastric mucosa.

CHAPTER 3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Gastric tissues:

All the specimens were drawn from the archival blocks of Manchester Royal Infirmary, which were formalin-fixed and paraffin-embedded. The terminology employed in this study was those of Laurence's classification (Laurence, 1965), which categorizes all gastric carcinomata into two groups: intestinal and diffuse types, and the WHO subclassification to describe the lesions in each section. All blocks were resectioned and stained with haematoxylin and eosin (H&E) for evaluation in order to review the original diagnoses. Detailed information upon the specimens is summarised in the table below.

Table 3.1 Summary of tissues used in this study

Tissues	Cases
Normal	15
Chronic Gastritis*	10**
Carcinomata	55
Intestinal Type:	40
Well-differentiated	11
Moderately-differentiated	10
Poorly-differentiated	19
Diffuse Type:	13
Simple type	8
Signet-ring cell type	5
Metastasis:	2
Total:	80

*: This refers to those slides that show only the lesions of chronic gastritis. **: There are 33 tumour specimens containing chronic gastritic lesions, which will be mentioned in later sections.

A total of 80 specimens (15 cases of normal gastric tissues as controls, 10 cases of chronic gastritis and 55 cases of different types of gastric carcinomata) were used in this

study. 7 specimens of duodenal mucosa were also examined to determine the staining patterns of duodenal tissue and particularly that of goblet cells. The actual number of cases of gastric tissue analysed varied from one lectin to another based upon the primary results and their further implications. All samples were re-identified and re-assessed carefully with the help of pathologists in the department and compared with the original diagnoses. Some specimens had more than one kind of lesion. Thus, chronic gastritis is commonly found in gastric carcinoma tissues and is usually accompanied by metaplasia and/or dysplasia, while in gastric carcinoma specimens, chronic gastritis, metaplasia as well as dysplasia are commonly found in the adjacent tissue cases. These lesions were treated separately in comparison with carcinoma lesions.

3.1.2 Lectin histochemistry

3.1.2.1 Lectins

A panel of twenty-seven biotinylated lectins was employed in the this study. The lectin histochemical staining procedure was followed by the method described and modified by Jones and Stoddart (Jones and Stoddart, 1986. See below for detailed procedures). Where necessary, ß-elimination and neuraminidase digestion were performed with slides to be stained by 18 and 10 lectins, respectively. Their origins and general sugar binding specificities are summarised in table 3.2.

Most lectins were obtained from the Sigma Chemical Co. Ltd (now Sigma-Aldrich, Poole, Dorset, BH17, 7TG, subject to St Louis, MO 63178, USA) and were used at concentration of 10 μg/ml as tested. MAA and SNA were bought from Boehringer Mannheim, UK [(Diagnostics & Biochemicals) Ltd, Belle Lane, Lewes, East Sussex, BN7, 1LG) and used at concentration of 50μg/ml. GNA, NPA, LFA and STA were from EY laboratories (San Mateo, CA 94401, USA) and were used at 10μg/ml working concentration.

All lectins were diluted with TBS (Tris Buffered Saline) containing 2% (w/v) calcium chloride to their working concentration.

Table 3.2 The origins and sugar binding properties of lectins

ACRONYM	ORIGIN	SUGAR SPECIFICITY
PSA	Pisum sativum	α-D-Mannose in non-bisected bi- /tri-antennary complex N-linked sequences, especially with core fucosylation
LCA	Lens culinaris	α-D-Mannose residues, similar but not identical to PSA
e-PHA	Phaseolus vulgaris	Bi/tri antennary, bisected complex N-linked sequences
l-PHA*	Phaseolus vulgaris	Tri/tetra-antennary, non- bisected complex N-linked sequences
GNA	Galanthus nivalis	Nonreducing terminal α-D-Mannose, preferentially mannosyl α1,3 mannose linkage
NPA	Narcissus pseudonarcissus	α-D-Mannose, preferentially mannosyl α1,6 mannose linkage
UEA-1*#	Ulex europaeus	α-L-fucosyl terminals, especially L-Fuc α1,2 Gal β1,4 GlcNAc β1-
LTA#	Tetragonolobus purpureus (Lotus tetragonolobus)	α-L-fucosyl terminals, especially where clustered or on core sequences of N-glycans
PNA/AHA*#	Peanut (Arachis hypogaea)	Gal β1,3 GalNAc α1> Gal β1,4 GlcNAc β1
ECA*#	Erythrina cristagalli	Gal β1,4 GlcNAc β1-
BSA1B4#	Griffonia simplicifolia (Bandercia simplicifolia)	Gal α1,3 Gal β1,4 GlcNAc β1-
SNA#	Sambucus nigra	NeuNAc α2,6 Gal/GalNAc-
MAA#	Maackia amurensis	NeuNAc α2,3 Gal β1-
LFA#	Limax flavus	NeuNAc α2-, NeuNGlycosyl α2-
VVA*#	Vicia villosa	GalNAc α1,3 Gal β1-
WFA	Wisteria floribunda	GalNAc α1,6 Gal β1-> GalNAc α1,3 Gal β1-
SBA*#	Glycine max	Terminal GalNAc α1,3->Gal α1-
PTA*#	Psophocarpus tetragonolobus	GalNAc-> Gal
MPA#	Maclura pomifera	Gal β1,3 GalNAc α1-> GalNAc α1-
DBA#	Dolichos biflorus	GalNAcα1,3(Fuc α1,2)Gal β1,3/4 GlcNAcβ1-
STA	Solanum tuberosum	(-4 D-GalNAc 1-)$_2$>3 Gal β1,4 GlcNAc β1-
LEA	Lycopersicon esculentum	(-4 GlcNAc β1-)$_2$»Gal β1,4 GlcNAc β1-
DSA*#	Datura stramonium	Gal β1,4 GlcNAc β1->(-4 GlcNAc β1-)$_2$
HPA*#	Helix pomatia	D-GalNAc
BPA*#	Bauhinia purpurea	β-D-Gal α1,3-D GalNAc-
CTA	Erythrina corallodendron	Gal β1,4 GlcNAc, esp in multiple branches
BSA-II#	Bandeiraea simplicifolia	D-GlcNAc

Note: '*' refers to Neuraminidase-digestion being carried out; while '*#' means both Neuraminidase-digestion and β-elimination being performed and single '#' refers to only the β-elimination reactions being performed. Underlined lectins were used for the first time in gastric tissues .

3.1.2.2 Avidin-conjugated peroxidase

Avidin-conjugated peroxidase was purchased from the Sigma Chemical Co. Ltd. It was used to bind to the biotinylated lectins and to act as the disclosing agent It was diluted with TBS to its work concentration of 5μg/ml.

3.1.2.3 DAB (3,3-diaminobenzidine tetrahydrochloride dihydrate)

DAB can be oxidised by H_2O_2 in the presence of peroxidase and to yield a brown coloured insoluble product at the sites where the enzyme is present. DAB was also obtained from the Aldrich Chemical Co. Ltd (Gillingham, Dorset, SP8 4BR, UK) and its solution was used at a standard concentration of 0.5mg/ml.

3.1.2.4 Eradication of endogenous peroxidase

For obtaining unambiguous results, it is necessary to remove endogenous peroxidase. The following reagents were used for this: methanol (400ml), 1N HCl (1.6ml) and 30% (v/v) hydrogen peroxide (2ml) were freshly mixed together. Slides were put into this solution for 30 minutes at room temperature (18°C). They were then rinsed in tap water, followed by TBS.

3.1.2.5 Pretreatment with crude trypsin

'Trypsinisation' serves as a procedure for exposing sugar groups hidden by the cross-linking of protein during formalin fixation, since it can digest these proteins. The preparation of the trypsin solution was undertaken by dissolving 300mg crude trypsin (type II, from porcine pancreas, Sigma Chemical Co.) and 300mg calcium chloride in 300ml TBS.

The TBS solution was pre-heated to 37°c and the trypsin and calcium chloride were then added. They were dissolved with stirring. Slides were pre-warmed in a solution of TBS only at 37°C, and were then transferred to the above solution for 15 minutes. The enzyme reaction was stopped by rinsing the slides in cold running tap water.

3.1.3. Neuraminidase treatment

Neuraminidase IV, for neuraminidase digestion was also obtained from the Sigma Chemical Co. Ltd.. It was derived from *Clostridium perfringens* and used at a working concentration of 10 μg/ml diluted in 0.01M sodium acetate buffer (pH 5.5). The solution was pipetted onto the section on each of the slides and care was taken to ensure that the sections were completely covered. Each slide was incubated for 1 hour at room temperature (18°C). The digestion was stopped by rinsing the slides in cold running tap water.

3.1.4 Reagents for β-elimination

Oligosaccharides O-glycosidically linked to serine or threonine via a-N-acetylgalactosamine are labile to alkali, which cleaves the O-glycoside to leave an unsaturated bond in the amino-acid. Under reducing conditions serine is converted to glycine and threonine to alanine, with the production of a sugar alcohol at the non-reducing terminus of the glycan. By comparing the lectin staining patterns of sections of the same tissue variously exposed and not exposed to conditions for β-elimination, the content of O-glycan in tissues and its contribution to lectin binding can be explored (Boyd, 1949).

Beta-elimination was performed using a reagent composed as follows: 50ml DMSO (Dimethylsulphoxide), 40ml distilled water, 10ml 99% (v/v) ethanol and 0.954g Potassium hydroxide (for 100 ml aliquot).

The above mixture was heated in a water bath to 45°C and the slides were incubated in the solution for 40-60 minutes. The alkali was then neutralised by washing the slides in 10mM HCl (1ml 1N HCl diluted in 100ml distilled water), with two changes, each of 5 minutes. The slides were rinsed in PBS (sodium phosphate buffer) pH 7.0 shortly then in water, followed by TBS (Downs *et al*, 1973).

3.1.5 Sialyl Lewis^a antigen and MUC-1 staining

In order to explore the relationship between lectin staining and mucin expression, anti-sialyl Lewis^a, and anti-MUC-1 antibodies were used. A mouse monoclonal antibody,

CA19-9, recognising sialyl Lewis[a], and an anti-muc-1 product monoclonal antibodies were purchased from Novocastra Laboratories Ltd (24 Claremont Place, Newcastle Upon Tyne, NE2 4AA, UK) in lyophilised form. The staining procedures are described in the method's section.

3.1.6 Other staining

Regents for H&E, PAS/Alcian Blue were introduced as follows:

(1) H & E (Haematoxylin & Eosin) staining

Mayer's haematoxylin solution was made with the following reagents: 1g Haematoxylin, 1000ml distilled water, 50g aluminium potassium sulphate, 0.2g sodium iodate, 50g chloral hydrate and 1g citric acid.

Haematoxylin, aluminium potassium sulphate and sodium iodate were dissolved in the distilled water, chloral hydrate and citric acid were then added. The mixture was boiled for 5 minutes, then cooled and filtered through a filter paper.

Eosin solution was made with 5g yellowish eosin, 1000ml distilled water and 0.1ml glacial acetic acid. The glacial acetic acid was mixed in the distilled water to make 0.01% (v/v) acetic acid solution, the eosin was dissolved in the solution and filtered.

(2) PAS / Alcian blue for acid and neutral mucins

The PAS (Periodic acid Schiff's) reaction was performed as follows: Periodic acid 1% (v/v)and Schiff's reagent which was made of 2g paraosaniline, 100ml 0.25M HCl, 3.9g of sodium metabisulphite and 2g of decolourising charcoal.

Alcian blue solution was made by dissolving 1g Alcian blue 8GX in 100ml 3% (v/v) acetic acid.

3.2 METHODS

3.2.1 H & E staining and PAS/Alcian blue staining

The purpose of these staining procedures was to achieve the recognition and subtyping of the tumour tissues and the comparison of the results with the original diagnoses. They

also give basic information as to the types of classical mucins present in various cell types in the mucosal layer. These conventional procedures were followed by lectin histochemistry.

3.2.2 Lectin staining procedures

3.2.2.1 Controls

Negative control slides were treated with an equivalent volume of TBS instead of biotinylated lectins in the following procedures.

3.2.2.2 Routine lectin staining:

All slides were pre-treated with 3% (v/v) APES (3-aminopropyl-triethoxysilane, the Sigma Chemical Co.) by following procedure. Firstly, the slides were rinsed in 95% (v/v) IMS for 2 minutes and air dried; secondly, they were placed into the APES solution for 30 seconds and then rinsed rapidly in acetone; finally, the slides were dried in the 50°C oven overnight.

Sections (4μm) were cut and mounted onto APES coated slides. After normal dewaxing and rehydration as above, the slides were processed by the following avidin-biotin peroxidase staining procedure of Jones and Stoddart (1986).

(1). Endogenous peroxidase activity in the sections was blocked with acidified methanolic hydrogen peroxide for 30 minutes. They were then rinsed briefly in tap water then in three changes of TBS for 5 minutes each.

(2). Trypsinisation: Slides were incubated for 15 minutes in 300ml of crude trypsin solution warmed to 37°C by a water bath. They were then washed in cold running water to stop the enzyme reaction, followed by three rinses in TBS, each for 5 minutes.

(3). Lectin binding: The slides were placed in a levelled wet-box after removal of excess TBS. Biotinylated lectins (10μg/ml) were carefully pipetted on, to cover every part of each section. Slides were then incubated 30 minutes at room temperature (18°C).

(4). Using TBS+$CaCl_2$ solution, lectin was washed away from each slide by using a

wash-bottle. The slides were then placed in racks vertically and rinsed in the same TBS plus calcium chloride for three changes, each of 5 minutes.

(5). The slides were drained of excess buffer onto a paper-towel and returned to the wet-box. Avidin-peroxidase solution (5mg/ml) was then pipetted onto each slide and was incubated at room temperature (18°C) for 60 minutes. Uncombined avidin-peroxidase was jet-washed away by means of a wash-bottle filled with TBS and this was followed by rinsing in TBS for three changes, and each of 5 minutes.

(6). Visualisation: The slides were developed in DAB solution for 5 minutes at 18°C. They were further rinsed in cold water to stop the reaction.
(7). Counterstain: The slides were put into 0.25% (w/v) methyl green for 30 seconds were then rinsed in tap water, dehydrated, cleared and mounted as above.

3.2.2.3 Neuraminidase digestion:

Neuraminidase can selectively spilt terminal sialic acid residues from oligosaccharide chains and expose the sub-terminal sequences for detection by lectin binding. Therefore, after digestion, any differences in staining patterns will provide additional information if there is any sialic acid present in the mucins.

Depending on the specificity of the individual lectin, 10 of the 27 listed in table 3.2 were chosen as appropriate to use in this analysis. The following steps were added after trypsinisation:

Neuraminidase IV solution (0.1 unit/ml in 0.2m acetate buffer pH 5.5 containing 1% (w/v) calcium chloride) was pipetted onto the slides and they were then incubated for 60 minutes at 37°C. The slides were rinsed in distilled water and washed in TBS for 3 changes each of 5 minutes. They then followed the rest of standard lectin staining procedure.

3.2.2.4 β-elimination:

To achieve β-elimination, after Trypsinisation, the following procedures were inserted:

(1). Slides were placed in 100ml of a 0.17M potassium hydroxide solution containing 50ml DMSO (Dimethyl-sulphoxide), 40ml distilled water, 10ml 99% (v/v) industrial methylated spirit (IMS) and 0.954g potassium hydroxide and incubated at 45°C in a water bath for 60 minutes.

(2). The slides were removed and neutralised by washing in two changes of 10mM HCl for 3 minutes each.

(3). The slides were then rinsed in 0.1M sodium phosphate buffer (pH 7.0) for 15 minutes.

(4). After rinsing shortly in distilled water, they were transferred to TBS. They then continued through the lectin staining procedure, as above.

CHAPTER 4 RESULTS

4.1 H & E STAINING

The newly H & E stained slides were carefully re-examined microscopically to evaluate the previous diagnoses and to determine the subtypes of the tumourous tissues. The overall observations were consistent with the previous description as in table 3.1.

In order to describe the stained parts of gastric mucosa, the following terms were used to refer to specific parts, as shown in figure 1: 'surface' means the top layer of mucosal epithelium, 'pit' means the part between the 'surface' and the glands (including the neck cell zone), 'glands' refers to the part from the neck zone to the bottom of glandular structures. Since gastric body mucosa possess these three distinctive layers and the surface mucin cells have identical features to the cells in the mucosa of the cardiac and pyloric areas, they were used as normal controls in this study (Owen, 1986) (Figure 1).

In addition to the tumours themselves, other pathological changes were also found in the same specimens. These included chronic gastritis, intestinal metaplasia and dysplasia of various degrees (Figure 2). Since they were generally considered to have a close relation to the gastric carcinogenesis and gastric carcinomata often occurred in the background of these lesions, they were often termed as 'pre-cancerous' or 'precursor' lesions. Thereby, these non-cancerous lesions will be referred as 'precursor' or 'precancerous' lesions in later chapters, as is common usage in the literature. Table 4.1 summarises these lesions in the adjacent mucosa of gastric carcinoma specimens.

Table 4.1 Metaplasia and dysplasia in the mucosa adjacent to carcinoma tissues

	Intestinal-type			Diffuse-type	
	Well[1] (8/11)	Moderately (8/10)	Poorly (15/19)	Diffuse (4/8)	Signet[2] ring(4/5)
CG*	8	8	11	3	3
MP**	8	7	8	2	2
Dys***	4	5	5	1	2
CG+MP	7	6	1	1	2
CG+Dys	4	5	1	1	1
CG+MP+Dys	4	5	1	1	2

[1]: Well-, Moderate, Poor and so on refer to the differentiation of the gastric carcinoma from high-grade to low-grade; [2]: Simple means the common histology of diffuse-type gastric carcinoma and signet-ring-type is the special type of diffuse gastric carcinoma (according to Laurence's Classifications) and here is separately dealt with. *: CG refers to chronic gastritis; **: MP to metaplasia; ***: Dys to dysplasia.

It appears from the table 4.1 that chronic gastritis and metaplasia are common findings in the adjacent or remaining mucosa of gastric carcinomata. They were more common in intestinal-type gastric carcinomata (27/31 cases) than in diffuse-type (6/8 cases). There are slight differences in occurrence of these accompanying lesions among the subtypes of the intestinal-type of gastric carcinoma. Well and moderately differentiated carcinomata are more likely to have chronic gastritis and metaplasia accompanying them than are the poorly-differentiated type. The same applies to the presence of dysplasia, which is more frequently found in well and moderately differentiated gastric carcinomata, but less frequently in the poorly differentiated type. In some cases, these three lesions could appear in the same mucosa.

These observations correspond to those of other authors and show the potential relation of these precancerous lesions to gastric carcinogenesis (Morson et al, 1980; Sipponen, 1981, Iida and Kusama, 1982; Ming et al, 1984).

4.2 PAS/ALCIAN BLUE STAINING

In order, first, to determine the gross characteristics of mucins in the different cells in the mucosal layer, this combined staining technique was performed.

The following staining patterns were found in the different tissues:

(1). In normal epithelium, magenta, the typical colour of staining for neutral mucins, was dominant in all control slides and the relatively normal parts adjacent to carcinomatous tissue.

(2). In intestinal metaplasia, goblet cells were stained dark blue, which was strikingly different from other cells in the mucosal layer, which showed a magenta colour. What is specially important, is that by this PAS/AB staining, some goblet cells in the intestinal metaplastic mucosa, which were invisible under HE staining, became much more recognisable, because the alcian blue outlined the cell clearly with dark blue.

(3). For metaplasia, this staining method did not show much difference between the gastropathy and normal mucosa. The metaplastic cells were mainly magenta in colour, as in normal cells, with slight variability in the staining intensity.

(4). Dysplastic tissues showed little concordance with the normal tissue and varied in colour, with various magenta, red, deep red, purple and blue staining. This staining pattern resembled that of the carcinomata

(5). Carcinomatous tissues generally showed blue staining, light or dark, but some differed with magenta, purple or mixed coloration. What is more, the adjacent tissue lying between the normal and the grossly neoplastic area usually showed a gradual transition of colour from magenta, through purple, to blue.

These results indicate a range of changes in the compositions and chemistry of gastric mucins in the different gastric lesions and, in particular, in gastric carcinomata. These changes ranged from slight to extensive. Thus, gastric mucins were probably undergoing a shift from neutrophil to acidophil, by the increase of sulphomucins, which was responsible for the colour changes detected by PAS/Alcian blue staining.

4.3 DIRECT LECTIN STAINING

The staining intensity was ranked semi-quantitatively by using (-) for negative, (+) for detectable, positive staining; (++) for moderately positive, to clear, detectable staining; and (+++) for strongly positive, to deeply brownish staining. Figures were given by calculating the percentages of positively stained carcinoma cells, in all specimens, in the areas densely populated with tumour cells.

In order to indicate the distribution of stain within or upon a cell, 'cytoplasmic' refers to the staining within the cytoplasm of cells and 'membrane' refers to the staining mainly on

or at the cell membrane. These terms were used to distinguish the dominantly stained parts. These assessment and recording methods were adopted in most of the content of this study, unless illustrated otherwise.

In most cases, each lectin produced a distinctive staining pattern, but some lectins also showed similar or even identical patterns to each other. The staining patterns of lectins in normal and precancerous gastric tissues were described individually as follows below.

PSA: Recognising non-bisected bi-/tri- antennary complex N-linked sequences

As shown in Figure 3, PSA stained surface epithelial cells very faintly or not at all, with a few supranuclear granules, but produced very clear staining from pit cells downwards deep into the area of glandular cells (++). In the pit, some neck cells were stained and most of gland cells showed staining in their cytoplasm. It was noted that there was a difference between chief cells and parietal cells, in that chief cells accounted for the mainly stained components, while parietal cells showed partial staining in nuclei and lumenal membrane and their cytoplasmic staining was hardly seen. Extracellular staining tended to be fairly strong (+++) as was that of blood vessel walls.

In the mucosa with gastritis, the staining pattern and intensity of PSA varied remarkably from the normal counterpart. The staining was usually decreased in intensity or was completely lost, while 2 out of 6 cases remained unchanged or slightly increased. Metaplasia and dysplasia showed similar patterns to those of gastritis with diminishing intensity of staining with increasing cellular abnormality. Goblet cells in metaplastic tissues were negative in all the specimens observed.

LCA: Recognising α-D-mannose, similar to PSA

LCA was observed showing similar staining patterns to that of PSA in normal (Figure 6) and non-neoplastic lesions. Surface cells were negative or showed weak membrane staining in only a few cells, while more pit cells were stained (+). Gland epithelial cells generally showed clear perinuclear staining (++), in which chief cells were the dominant cells with cytoplasmic staining and parietal cells stained faintly in their nuclear membranes. Stromal staining was strong, as was that of the endothelial cells of the blood vessels. Similarly, in the precursor lesions, staining intensity was dramatically decreased or lost in most cases and metaplastic goblet cells showed no staining at all.

e-PHA: Recognising bi-/tri-antennary, bisected complex N-linked sequences

This lectin displayed a very strong staining in most gastric tissues of all types (Figure 8). In normal tissues, the surface epithelial cells showed slightly stronger staining than the glandular epithelial cells. The staining was mainly shown on the membrane and in the supranuclear region, so that the cells were outlined clearly. In glands, some cells showed staining of the nuclear membrane and most cells gave no cytoplasmic staining. There was no marked difference in staining between chief and parietal cells, since parietal cells showed a similar staining to that of chief cells. Stroma tended to stain strongly with e-PHA, which led to difficulty in distinguishing the border between it and glands.

In chronic gastritis, metaplasia and dysplasia, the staining patterns of the mucosa were very similar to these of normal tissues, though a variation in staining intensity was noted

in some cases. The goblet cells in metaplastic tissue were weakly stained by e-PHA.

l-PHA: Tri-/tetra- antennary, non-bisected complex N-linked sequences

This differed from its isolectin, e-PHA, in that it showed relatively weak staining in the surface epithelial cells of normal gastric tissues (Figure 10). Some surface cells were positive on their lateral and surface membranes. The neck cells in the pit area produced stronger staining than surface cells and displayed dark brownish granules in their supranuclear regions. Most gland cells did not show detectable stain. Stromal staining was seen, which was strongly positive in the walls of blood vessels with a clear linear staining.

In chronic gastritic mucosa, staining was increased in the lumenal membrane, in comparison with that of the normal controls. In metaplastic and dysplastic tissues, the staining tended to be stronger than that of the normal counterparts. Goblet cells were clearly shown by the intense staining of their mucin in the cytoplasmic vacuoles.

GNA: Non-reducing terminal α-D-mannose, preferentially mannosyl α1,3 mannose linkage

This lectin gave a distinctive staining pattern (Figure 13). It gave little detectable reaction in the surface cell layer and in the pit cells, but stained the glandular epithelial cells strongly. It had some selectivity for chief cells rather than parietal cells. The stroma was negative, so that a bright contrast was produced by positively stained epithelial cells and the negative stromal background. Blood vessels also showed no staining. Some plasma

cells exhibited detectable positivity.

In gastritic, metaplastic and dysplastic tissues, the staining was diminished in intensity or completely lost. Only a few cases showed scattered, positively stained, epithelial or gland cells. Goblet cells in metaplastic areas were not reactive with this lectin.

NPA: α-D-mannose in mannosyl α1,6 mannose linkage

This lectin did not stain surface cells and pit cells (Figure 15). Gland cells were generally positive in most. The positive cells showed cytoplasmic or supranuclear, granular, staining patterns. In positively stained gland cells, parietal cells showed preferentially strong staining, while chief cells were usually weak or negative. Stromal tissues gave only very faint staining or no staining at all, as did blood vessels.

The staining was conspicuously diminished or absent in all cases of potentially precancerous lesions. Accordingly, goblet cells in intestinal metaplasia lost their staining as well.

UEA-1: α-L-fucosyl terminals in the L-Fuc α1,2 Gal β1,4 GlcNAc β1- sequence

This was one of two fucose-specific lectins used in this study. It gave a staining pattern (Figure 17) which was characterised by a pattern in which a variable proportion of epithelial cells above the neck area showed detectable positive staining. The neck zone and the upper two-third of the glands beneath had no staining, but the most basal third of the gland cells showed strong cytoplasmic staining, in which chief cells were the main

positive population (Figure 18). This is similar to the patterns produced by the lectins NPA and LTA (see above and later sections). The stroma was generally negative. Some blood vessel walls appeared to show clear-cut, linear, endothelial staining.

In gastritis, the epithelial cells, as a whole, tended to be strongly positive, with slight variation in staining intensity. The epithelial cells in metaplasia (Figure 19) gave a similar appearance to those in gastritis, with strongly and clearly positive goblet cells in-between. In dysplasia, the cells also showed strong positivity.

LTA: α-L-fucosyl terminals, especially clustered or on core sequences of N-glycans

This fucose-binding lectin yielded a staining pattern which was very similar to that of UEA-1, but with some obvious variation (Figure 22). It did not stain superficial cells as well as pit cells above the neck zone, but most glands were intensely stained, with the stain being cytoplasmic. The chief cells were the dominant component and had intense staining with LTA, while parietal cells, in contrast, showed very weak staining. Stromal staining varied between negative or weakly positive.

In chronic inflammatory tissues, the staining appeared to be increased in the epithelial cells, which contained stained cytoplasmic granules. In metaplastic and dysplastic tissues, the staining intensity of epithelial cells showed no obvious difference from their normal counterparts. Goblet cells in metaplastic tissues were positively stained (Figure 23).

PNA/AHA: Gal β1,3 GalNAc α1> Gal β1,4 GlcNAc β1-

Most gastric mucosal cells, including surface cells, pit cells and the upper parts of most glands, showed very strong, positive staining, mainly in cytoplasm. Slight variations occurred in some glands, in which part of cells were not stained by PNA (Figure 25). The basal parts of the glands generally were negative. This lectin appeared to show selective staining of chief cells, which were strongly positive (Figure 26), while parietal cells were usually negative or only weakly stained. Stroma was completely negative with PNA, so producing a sharp contrast between epithelial cells and their surrounding stroma.

Epithelial cells in chronic gastritis generally showed a diminution in staining intensity as compared with normal tissues, although the extent of this varied in individual cases. The phenomenon became more apparent in metaplasia, in which most cells showed sharply decreased staining intensity. Goblet cells were negative. Dysplastic epithelial cells also exhibited both strong staining and an irregular staining pattern (Figure 27).

ECA: Recognising the Gal β1,4 GlcNAc β1- linkage

As showed in Figure 28, surface and pit epithelium showed strong cytoplasmic staining. Most cells in the neck zone were clearly stained. Glandular epithelial cells showed variations: chief cells were positive, while parietal cells were negative (Figure 29).

In gastritis, metaplasia and dysplasia, as compared with the staining of normal counterparts, epithelial cells tended to lose their reactivity with ECA, so that most cells

were either faintly stained or completely negative. Goblet cells showed cytoplasmic staining which highlighted cells clearly.

BSA1-B_4: Sugar specificity: Gal α1,3 Gal β1,4 GlcNAc β1-

This lectin selectively stained surface and pit epithelium very strongly (Figure 32), while most of the upper parts of the glands were negative, with the bottom part being positive. Staining was mostly cytoplasmic and was predominantly in chief cells. Neck cells and parietal cells did not react with this lectin. Stroma and blood vessels were negative. A very bright contrast could be seen between stained epithelium and the negative stromal background.

In precursor lesions, the staining of majority of epithelial cells showed diminished staining, but variations were also observed from strongly positive to negative (Figure 33). Most gland cells were also negative, except for a few positive foci. In metaplasia, the staining patterns of gastric mucosa differed between specimens. Most cells showed partial or diminished staining. Goblet cells in the metaplastic tissues, however, produced a consistent and similar cytoplasmic staining. Dysplasia was similar in that the epithelial cells population showed great variations.

SNA: NeuNAc α2,6 Gal/GalNAc- linkage

This is a lectin considered to be specific for N-acetyl neuraminic acids in α2,6 linkage to galactose or 2-deoxy, 2-acetamido-galactose. In gastric mucosa (Figure 34), the surface and pit cells were negative, while a proportion of cells in the neck zones and most cells in

the upper parts of glands (mainly parietal cells) were strongly stained in their cytoplasm. The segments representing the bottom thirds of the glands (mainly chief cells) were negative. The stroma was universally strongly stained, but the blood vessels did not show staining.

In the precancerous lesions, most cases showed a general reduction or loss of staining with SNA, especially in the cells of the glands. The strongly stained stroma made the weakly stained or non-stained epithelium readily recognisable.

MAA: Sugar specificity: NeuNAc α2,3 Gal β1- linkage

This lectin is specific for N-acetyl neuraminic sialyl acid in α2,3 linkage to galactose and so differs from SNA which is specific for the α2,6 linkage.

It was found that the normal gastric mucosa (Figure 36) was almost all unstained, except for a few positive cells scattered in surface and pit layers. The cells in neck zone and in glands were also rarely and scarcely stained. Stroma was comparatively strongly stained.

In gastritis, metaplasia and dysplasia, staining was generally faint and varied slightly. Some cases showed scattered positive cells, focally, in different layers of mucosa. Goblet cells were negative in all cases.

LFA: Recognising NeuNAc- and possibly NeuNGlycolyl- saccharides

In normal gastric tissues (Figure 40) all mucosal layers were negative, except for a few,

scattered, gland cells. Stroma and blood vessels were strongly stained (++), especially vascular endothelium, which was clearly outlined by LFA.

In gastritis, metaplasia and dysplasia the epithelial cells and gland cells were mostly positively stained in the cytoplasm. Goblet cells had strong positive cytoplasmic stain (Figure 41), which was consistent with the staining of the goblet cells in the duodenal mucosal layer.

VVA: GalNAc α1,3 Gal β1- linkage

This lectin yielded a very distinctive staining pattern in normal gastric tissues (Figure 43). Only surface and pit epithelial cells were clearly stained at grade (++) in their cytoplasm, while gland cells were very rarely positive. Stroma was negative in all layers, including the smooth muscle. The stained epithelial cells appeared very striking against the negative background.

In gastritis, the stained parts were still limited to the surface and pit areas, but the intensity of staining was greater than in normal controls and more gland cells were positively stained. Metaplastic epithelium (Figure 44) showed a similar staining pattern to the cells in gastritis, in which goblet cells were positive. Dysplastic cells also gave strong staining.

WFA: GalNAc α1,6 Gal β1->GalNAc α1,3 Gal β1- linkage

All epithelial cells in the normal mucosa were very strongly positively stained at grade

(+++) in their cytoplasm, irrespective of their locations (Figure 47). Among the glandular cells, staining of the chief cells was dominant, while parietal cells were sparsely and only faintly stained. Again, WFA did not stain stroma in any layers.

In all precursor lesions, the staining patterns of WFA remained very similar to their normal counterpart, which showed strongly stained mucosal epithelium against a clear background. The goblet cells in metaplasia were also positive.

SBA: Specific for terminal GalNAc α1,3-> Gal α1- linkage

SBA had a similar staining pattern to VVA in normal mucosa and only epithelial cells showed reactivity with it (Figure 50). It stained surface and pit cells intensely in their cytoplasm. Positive cells in the neck zone and glands were very few and scattered and were mostly parietal cells. No stromal staining was seen in any layer.

Gastritis, metaplasia and dysplasia displayed a diminished staining, in comparison to that of normal mucosa, although some cases showed variations of staining (Figure 51). It was noted that the number of stained cells in the neck zones and glands was increased in comparison to controls. The cytoplasm of goblet cells was clearly shown (Figure 50).

PTA: GalNAc->Gal- linkage

This lectin produced a distinct staining pattern (Figure 54), in that most epithelial cells, from the surface to the bottom of the glands, were not stained. Only in a few, scattered cells, in the neck zone and in the glands, was there cytoplasmic stain. The positive cells

of glands were mainly chief cells. The stroma was either unstained or only very faintly stained. Some blood vessel walls were selectively stained.

All precancerous lesions showed an increased staining intensity in that an increased number of surface and pit cells yielded a positive reaction, which was of grade ++. The staining intensity of gland cells varied, in that part of cells had diminished or absent staining in their cytoplasm. Goblet cells were clearly shown by PTA staining in this case (Figure 55).

MPA: Specific for Galβ1,3 GalNAc α1->GalNAc α1- structures

This lectin was not very informative, since it stained every part of all mucosal layers intensely (Figure 58). On careful observation, it was found that the stroma was stained a little more lightly than the epithelial cells. It was noted that chief cells were much more strongly positive than parietal cells (Figure 59), so that MPA can readily distinguish between these two kinds of cells. Stroma was also strongly stained, which made it difficult to define the boundary between it and epithelium.

There was no significant difference between the precursor lesions and their normal counterparts or among themselves, although staining intensity tended to decrease in all of these lesions. The goblet cells in metaplastic tissues were clearly shown (Figure 60).

DBA: For GalNAc α1,3 (Fuc α1,2) Gal β1,3/4 GlcNAc β1-

In normal gastric mucosa (Figure 62), the cytoplasm of the surface and pit cells was

clearly stained. Positively stained cells were dotted throughout the neck zone and glands. Most of these were parietal cells. No stromal staining was observed.

In chronic inflammatory lesions (Figure 63), the affected epithelium showed variations in the staining patterns: some still were positively stained as intensely as their normal controls, but others showed diminished stain or lost reactivity to DBA. Metaplastic and dysplastic lesions showed very similar staining changes to those in gastritis. Goblet cells in metaplasia were positive (Figure 64), though not so strongly so as other cells in the epithelium.

STA: Specific for (-4 D-GalNAc 1-)$_2$>3 Gal β1,4 GlcNAc β1-

As with MPA, this lectin stained all components in the gastric mucosa intensely (Figure 65), though the staining of the surface and pit epithelial cells was slightly less strong than that of the gland cells, in which chief cells were strongly stained rather than parietal cells (Figure 66). Stroma was strongly positive in all cases.

It is noteworthy that the staining of the epithelial cells in gastritis appeared to be reduced or diminished (Figure 67), while in metaplasia the staining of epithelial cells was rarely seen. In dysplasia the staining of epithelial cells was almost absent.

LEA: For (-4 GlcNAc β1-)$_2$>>Gal β1,4 GlcNAc β1-

There was a general positive staining in the mucosal layer, but with particular differences (Figure 69). The surface and pit cells (+) were less intensely stained than glandular

epithelial cells (++). Not all gland cells were as intensely stained as parietal cells, since the chief cells were much more lightly stained. This contrast is distinctive enough to discriminate between these two types of cells. The bottom part of glands (mainly chief cells) were usually unstained. The stroma was faintly positive.

In most cases of gastritis, the epithelial staining tended to be decreased. The metaplastic tissues with goblet cells were generally negative. Dysplasia showed a similar staining patterns to those of the gastritis and metaplasia in which only few cells had supranuclear granular staining.

DSA: specific for Gal β1,4 GlcNAc β1-> (-4 GlcNAc β1-)$_2$

Normal mucosal surface and pit cells were generally positive (+) in their cytoplasm (Figure 71). Cells in neck zone and upper glands were clearly and partially stained (++). The stained cells morphologically appeared to be parietal cells. Stroma was strongly stained in most specimens.

Staining in precursor lesions varied, with a trend towards diminution in the epithelium (Figure 72), as compared with that in normal cells. Cases of metaplasia and dysplasia were uniformly negative.

HPA: Specific for D-GalNAc

The surface and pit epithelial cells were seldom stained by HPA, but the cells in glands were stained very clearly (++) in their cytoplasm (Figure 74). Chief cells were dominant,

while parietal cells were generally faint (Figure 75). HPA also bound to blood vessels and red blood cells (RBC) in some cases. Stroma was usually negative.

In gastritis (Figure 76), the epithelium showed apparent variations, in which some cells displayed an increased staining while others showed lessened stain or had lost staining altogether. Similar change occurred in metaplastic tissues. The cytoplasm of goblet cells was strongly stained (++), which made them easy to recognise. Cases of dysplasia were also of variable staining, as were those of metaplasia.

BPA: Specific for D-Gal β1,3 GalNAc α-

It was observed (Figure 79), that BPA faintly stained a majority of surface, pit and neck zone epithelial cells, specifically in their supranuclear regions. Gland cells, in contrast, showed very strong staining and, among them, the chief cells were dominant (Figure 80), while parietal cells had only a faintly positive stain, which was limited to their surface membranes. Stroma was positively stained (++).

All precancerous lesions showed very similar staining patterns to one another. They all gave decreased or diminished staining or no staining in their lining cells. Some of the goblet cells in the metaplastic tissues were positive in the cytoplasm.

CTA: For Gal β1,4 GlcNAc-, especially in multiple branches

In normal tissues, only surface epithelium was stained (rather weakly), while the epithelial cells in other layers, including the neck zone and glands, were negative. Stroma was not stained (Figure 82).

In gastritis, metaplasia and dysplasia, the epithelial cells tended to lose their staining and, in metaplasia, goblet cells were completely negative.

BSA-II: D-GlcNAc-

This lectin gave a very characteristic staining pattern in normal gastric tissues (Figure 84). The surface and pit cells were negative. In contrast, most gland cells were strongly cytoplasmically stained (+++). Again, the positive cells were mainly chief cells, and parietal cells were only faintly stained on their membranes (Figure 85). No stromal staining was observed in any tissue. This produced a very sharp contrast in which positive parietal cells dominated.

In precancerous lesions, there was still no apparent positive staining in the epithelial cells above the neck zone. The staining intensity of gland cells was marginally diminished. Some cells in the neck zones showed faint staining.

In summary, a few lectins stained every component of the gastric mucosal layer or its stroma very strongly or identically, such as ePHA, MPA, ECA and STA. What is important is that there is a considerable numbers of lectins which showed very distinctive staining patterns in various gastric tissues, including normal controls and potentially precancerous lesions. Those lectins, which selectively stained all mucosal epithelial cells rather than stromal tissues, or one or more than one kind of the mucosal epithelial cells, are potential markers for gastric mucosal cells. The table 4.2 summarises the staining patterns of normal and precancerous pathological gastric tissues. The table 4.3 is a further summary of those lectins used in this study which showed selectivity for individual components of gastric mucosa.

Table 4.2 The direct lectin staining of normal and precancerous lesions

Lectin	EC	Pit	Gland	Stroma	BV	CG	MP	Dys
PSA	-	-	++	+++	+++	↓↑	↓↑	↓↑
LCA	-	+	+	++	-	↓	↓	↓
cPHA	++	+++	+++	+++	+++	=	=	=
lPHA	+/-	+	+	+	-	↑	↑↑	↑
GNA	-	+/-	++/c>p	-	-	↓	↓	↓
NPA	-	-	++/c>p	-	-	↓	↓	↓
UEA-1	-/+	-	+++/c>p	-	-	↑↑	↑G	↑
LTA	-	-	+++	-	-	↑	=	=
PNA	+++	+++	+++/c>p	-	-	↓	↓↓	↓
ECA	++	++	++/c>p	++	++	↓	↓	↓
BSA1B$_4$	+++	+++	-	-	-	↑↓	↑↓	↑↓
SNA	-	-/+	-/++	++	++	↓	↑↓	↑↓
MAA	-/++	-/++	-/++	-	-	↑↓	↑↓	↑↓
LFA	-	-	-/+	++	++	↑	↑↑G	↑
VVA	++	++	-	-	-	↑	↑	↑
WFA	++	++	++/c>p	-	-	=	=	=
SBA	+++	+++	-/+	-	-	=	=	=
PTA	-	-/+	-/+	-/++	-/++	↑	↑	↑
MPA	+++	+++	+++/c>p	+++	+++	=	=	=
DBA	++	++	-/++	-	-	↑↓	↑↓	↑↓
STA	++	++	++	++	++	↓	↓↓	↓↓
LEA	+	+	++/-	+	+	↓	↓	↓
DSA	-/+	-/+	++/-	++	++	↓	↓	↓
HPA	-/+	-/+	++/c>p	-	+	↑↓	↑↓G	↑↓
BPA	-	-	++/c>p	++	++	=	=	=
CTA	+	-	-	-	-	↓	↓	↓
BSA-II	-	-	++/c>p	-	-	=	=	=

Notes: If a '-' proceeds a '+', such as '-/+', this means that most cells were stained negatively, and vice versa. A '↑' refers an increase of staining, while a '↓' refers a decrease of staining. 'G' means that goblet cells were stained strongly or remained stained. 'c>p' refers to chief cells were stained more intensely than parietal cells and vice versa.

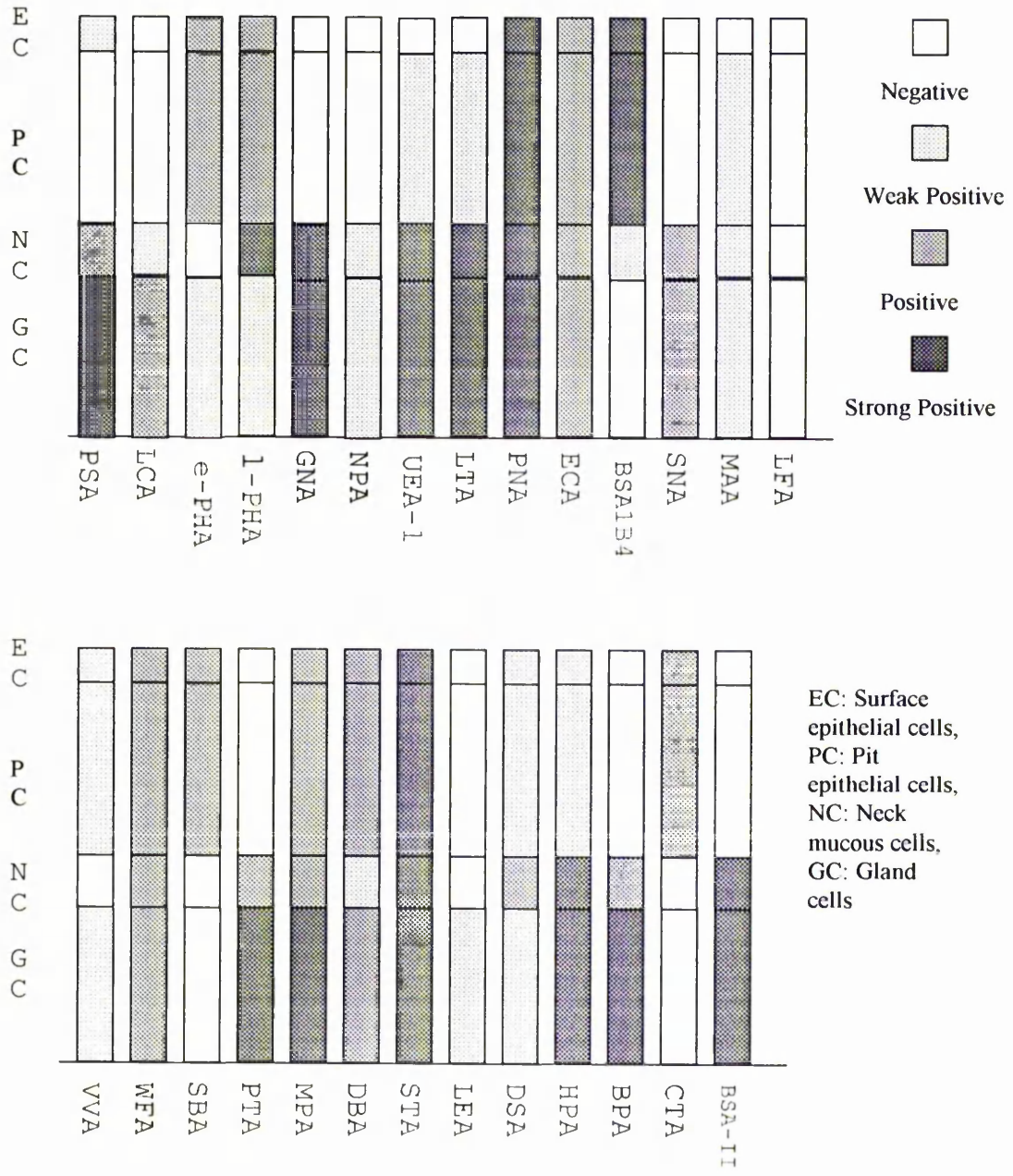
Table 4.3 Lectins showing selectivity in normal tissue

Components in Mucosal layer	Staining Intensity	
	Weak or Negative	Strongly Positive
Surface Epithelial cells	PSA, LCA, l-PHA, GNA, NPA, UEA-1, LTA, SNA, MAA, LFA, PTA, DSA, HPA, BPA, BSA-II	e-PHA, PNA, ECA, BSA1-B_4, VVA, WFA, SBA, MPA, DBA, STA, LEA, CTA
Pit Cells	GNA, NPA, UEA-1, LTA, SNA, MAA, PTA, DSA, HPA, BPA, CTA, BSA-II	PSA, LCA, e-PHA, l-PHA, PNA, ECA, VVA, BSA1-B_4, WFA, SBA, MPA, DBA, STA, LEA
Gland Cells	BSA1-B_4, MAA, LFA, VVA, SBA, PTA, DBA, CTA	PSA, LCA, e-PHA, l-PHA, GNA, NPA, UEA-1, LTA, PNA, ECA, SNA, WFA, MPA, STA, LEA, DSA, HPA, BPA, BSA-II

Notes: *: the epithelial cells refer to the surface cells including neck cells above the mucosal glands, sinc they usually show consistent or similar staining patterns. **: Glands here reflect all structures consisting of parietal cells, chief cells and other kinds of cells. ***: The intensity of staining grouped into two grades: Weaker Positive refers to microscopically visible of the staining as recorded as 1, while strongly positive refers to obvious positivity as recorded as grade 2 and 3.

The diagram 4.1 below provides a more direct overview of the staining of lectins in normal gastric mucosal cells. From this chart, it is evident that some lectins showed preferential affinity to epithelial cells in different parts of the gastric mucosal epithelial cells by their intense staining being focused on specific areas of the mucosa. Some of the lectins did not exhibit such a tendency and all parts of the mucosal epithelia were either all strongly stained or all weakly stained or even negative.

Figure 4.1 The staining patterns of normal gastric mucosa



Based on the observations above, it can be tentatively proposed that there are three groups of glycans expressed on various types of gastric mucosal cells.

One group is that the glycans with specific glycosyl residues are mainly expressed in mature cells or cells towards maturation, other than primitive cells (i.e. cells in neck

zone). These glycans can be detected by several lectins, which included $BSA1B_4$, SBA and CTA. There are other lectins which also showed stronger staining on surface and pit epithelium, as well as gland cells than neck cells. This included e-PHA, VVA and DBA. Hence, some glycosyl residues are more likely associated with the differentiation and maturation of gastric mucosal cells.

Another group is that some glycans are mainly present in immature mucosal cells, especially the cells in the neck zone. These glycans contain glycosyl sequences by the following lectins, including LCA, GNA, NPA, UEA-1, LTA, SNA, PTA, BPA and BSA-II. The glycans also contain the sugar residues which can be detected by MAA and HPA, since MAA stained faintly on a few mucosal cells which were mainly the cells in and around neck zone. HPA showed much stronger staining of cells in neck areas and downwards that it did on surface and pit cells.

The third group of glycans appear consistently to be synthesised through a cell life, from primitive cells to matured cells. They retain certain sugar sequences which can be detected by lectins PNA, ECA, MPA, STA and DSA.

Therefore, lectins which detect the changes in expression of these glycans in gastric carcinogenesis can be very useful indicators in determining the histogenesis and progression of a malignancy.

4.4 LECTIN STAINING AFTER NEURAMINIDASE DIGESTION

Based upon the results of direct staining, twelve lectins were selected for this procedure, in order to explore whether there were any sialyl termini attached to subterminal glycans

which the lectins could detect after their exposure by neuraminidase. The results are described as follows.

l-PHA: There was no marked change in terms of the stained parts and/or staining intensity, when compared with the results of direct staining. Generally, the staining tended to be slightly decreased in all components of the gastric tissues. Epithelial cells on the surface and in the pits, as well as some gland cells were focally stained (+ or ++). Stroma remained strongly positive.

In gastritis, metaplasia and dysplasia, the staining intensity appeared a little fainter after neuraminidase treatment, but variations in intensity among cases were seen. Goblet cells were not affected by neuraminidase digestion.

UEA-1: It appeared that neuraminidase digestion did not change the staining distribution and intensity given by this lectin in any normal gastric tissues, although a slight decrease of staining was observed in the precancerous lesions. The epithelial cells in chronic gastritic tissues, metaplasia and dysplasia were less frequently stained than by direct staining, but the goblet cells remained unaffected in most cases.

PNA: There was a general decrease in staining intensity in all types of gastric tissues, and in particular in the normal surface and pit epithelial cells. The extent of the reduction was of about one rank in grading. A very distinctive change was that the stroma became strongly stained, whereas it was nearly unstainable directly. Preneoplastic tissues also had a tendency to diminished staining after neuraminidase treatment. Goblet cells remained unstained after this pre-treatment in all cases.

ECA: As with PNA, this lectin diminished in its staining intensity in normal tissues. The epithelial cells of the surface and pits were paler than in normal controls, a proportion of these lost their staining. There was no change in cells in neck zones and in glands.

All precancerous lesions remained essentially unaltered after neuraminidase digestion, though a slight increase or decrease of staining intensity was noticed in some cases.

SNA: There was a general, marked decrease of staining in all mucosal cells, as compared with that in normal tissues. Since surface and pit cells were not reactive to this lectin, this diminution of staining intensity was mainly seen in neck and gland cells. Chief cells were still the predominantly stained cells in glands while most parietal cells were unstained. Stroma also showed lessened staining in most cases.

Precancerous lesions displayed similar staining patterns to their normal counterparts, but were much less intensely stained.

MAA: Neuraminidase digestion changed the staining patterns of normal mucosal cells to some extent. In comparison with the rare staining of epithelial cells by direct staining, those surface epithelial cells became almost unstained, while the pit and gland cells gave decreased, but still variable, staining, with some positive and some negative cells. Stromal tissues appeared not to be affected by this treatment. Gastritic, metaplastic and dysplastic tissues showed variable staining among the mucosal cells after this treatment, some showed an increased staining while some showed diminution and others remained unaffected.

VVA: Neuraminidase digestion affected those cells that were strongly stained by the direct procedure. Most cells showed a notable decrease in staining intensity, especially of the surface and pit cells. Gland cells were relatively unaffected by this pre-treatment. No stromal staining was seen after the digestion.

Mucosal cells from cases of gastritis showed variations in staining intensity, in that some were still strongly positive while others lost some staining. Metaplastic tissues had similar staining patterns to the inflamed, but, in them, goblet cells showed no sign of diminution or loss of staining. In dysplasia, epithelial cells were slightly less stained.

WFA: Neuraminidase digestion dramatically reduced the staining intensity and the number of positive cells in most normal and tumourous mucosal tissues. The surface epithelial cells showed only weak staining (+), compared with strong positivity (++) by routine staining. Most pit cells lost their staining. Only a proportion (about 25%) of the gland cells showed positive staining and those were mainly parietal cells Stromal tissue also lost its staining intensity.

Gastritis and other precursor lesions generally showed a tendency to loss of staining after neuraminidase digestion, although some variations were observed between cases. Goblet cells in metaplastic tissues were still positively stained.

SBA: Neuraminidase digestion dramatically affected the staining intensity of SBA on normal surface and pit cells, in comparison with the direct method. These cells showed a sharp decrease of staining positivity from (+++) to (+). Gland cells were not affected by

this pre-treatment and were still negative in all cases. No stromal staining was seen.

Staining in gastritic, metaplastic and dysplastic tissues was also clearly decreased in almost all cells from (+++) to (+). Some eventually lost their staining. Most goblet cells lost part or all of their staining.

DSA: The staining intensity of DSA was enhanced after this treatment. Both cells and stroma were extremely strongly stained, although this lectin had already stained these components strongly by the direct method. This increased staining made it difficult to distinguish the borderline between epithelial cells and their surrounding stroma. Similar increment of staining occurred in those precursor lesions.

HPA: It appeared that the pre-treatment had only a slight effect on the staining with this lectin. In normal gastric tissues, the staining intensity was a little stronger than that given by directly stained controls. The changes were mainly in the surface and pit cells, while gland cells seemed not to be affected by this enzyme.

Gastritic, metaplastic and dysplastic tissues were little affected by this treatment and still showed the same viability in staining intensity and patterns as seen previously. Goblet cells were also positive (++) in these cases.

In summary, although most lectin staining was not dramatically affected by the neuraminidase digestion, some lectins did show changes in their staining intensity, as illustrated in table 4.4.

Table 4.4 The results of routine lectin staining after neuraminidase digestion

Lectin	EC	Pit	Gland	Stroma	BV	CG	MP	Dys
IPHA	=	=	=	=	=	↓	↓	↓
UEA-1	=	=	=	=	=	↑↓	↑↓	↑↓
PNA	↓↓	↓↓	↓	↑	-	↑↓	↑↓	↑↓
ECA	↓	↓	=	=	=	=	=	=
SNA	↓	=	↓	↓	↓	↑↓	↑↓	↑↓
MAA	↓	↓↑	↓↑	=	=	↑↓	↑↓	↑↓
VVA	↓↓	↓↓	-	-	-	↓↑	↓↑	↓↑
WFA	↓	↓↓	↓	↓	↓	↓	↓	↓
SBA	↓↓	↓↓	=	-	-	↓	↓	↓
DSA	↑↑	↑↑	↑↑	↑	↑	↑	↑	↑
HPA	↑	↑	=	-	=	=	=	=
BPA	=	=	=	=	=	=	=	=

Notes: A '↑' refers an increase of staining, while a '↓' refers a decrease of staining. '=' refers to no much change after the digestion.

4.5 RESULTS OF β-ELIMINATION

Among eighteen lectins selected for this pre-treatment, a few did not stain, while most others showed results similar to those of direct staining. Some lectins exhibited a slight difference either in staining intensity or in distribution, which are described below.

UEA-1: In comparison with direct staining, surface epithelial cells remained negative and the pit cells were faintly positive. Staining with this lectin was notably susceptible to β-elimination, which decreased both the intensity and the number of positive normal gastric gland cells. The staining of stroma and blood vessels was either slightly decreased or totally lost.

The staining intensity in cases of gastritis, metaplasia and dysplasia remained strongly positive, some cases showed an obvious increase of staining intensity of surface epithelial cells after β elimination.

LTA: The staining pattern of this lectin was changed by β-elimination. The surface epithelial cells tended to be positively stained where they were negative before, while pit cells remained unaltered. The staining of gland cells was sharply decreased in most cases, in contrast to its previous strong reaction. Stromal tissues remained unaffected.

In precursor tissues, the staining of surface and pit cells was similar to that before β-elimination, but the gland cells showed a clear-cut diminution of staining. Goblet cells were still clearly stained in metaplastic tissues.

PNA (AHA): Staining with this lectin appeared to be insensitive to β-elimination, since it produced staining patterns of all the elements in the mucosal layer which were almost identical to those seen with the direct method. All epithelial cells were still strongly stained and stromal components were generally negative, as before.

ECA: The staining of all types of cells with this lectin seemed to be unchanged by the β-elimination pre-treatment. The surface and pit cells were still clearly stained. The only subtle change observed was that the staining intensity of gland cells slightly decreased. Stromal tissues showed a similar change to that of gland cells.

The precancerous lesions did not show alteration to their staining patterns and intensity

after this pre-treatment.

BSA1-B_4: This did not show any alteration on β-elimination in the surface and pit cell layers. A slight decrease of staining intensity occurred in the gland cell layer. Stroma and blood vessels remained unaffected by the treatment. The staining in gastritic, metaplastic and dysplastic tissues still showed the same decreased intensity relative to normal controls as found before.

SNA: All staining patterns and the intensity of staining of mucosal components with SNA remained as they were despite β-elimination.

MAA: The general appearance of staining with MAA was altered by β-elimination. The staining of surface epithelial cells in the normal mucosal layer was clearly increased in most specimens, with obvious cytoplasmic positivity. The pit cells and gland cells remained variously strongly stained or negative. Stromal tissues showed clear staining.

The staining in gastritic, metaplastic and dysplastic tissues showed variations similar to that of direct method, in their intensity after β-elimination, some epithelial cells showed an increased staining while some showed diminution or lost their staining.

LFA: This lectin did not showed no difference in comparison with the direct staining, both in normal and precancerous pathological tissues.

VVA: Pre-treatment by β-elimination did not greatly change the patterns and intensity of

staining of the normal and pathological gastric tissues. In general, the surface epithelial cells and pit cells still showed clear cytoplasmic staining, while gland cells showed a slight decrease in their stain intensity. Stromal tissues remained unstained.

WFA: The staining patterns of all components in the normal mucosal layer were unaffected by β-elimination. However, some changes in staining intensity were observed after the treatment, with a general slight decrease in the cytoplasmic stain. The stroma and blood vessels remained unaffected.

In gastritic and metaplastic tissues, staining intensities were decreased by about one rank, but the cytoplasmic granules were still well shown. Dysplastic tissues appeared to show no change on β-elimination.

SBA: There were alterations in the staining patterns and stain intensity with this lectin after β-elimination. The staining intensity of the surface epithelial cells was dramatically decreased from strong positivity (+++) to weakly positive (+). Only a few pit cells were faintly stained by the direct lectin staining, but they showed a strong cytoplasmic staining after β-elimination. Neither gland cells nor the stroma showed change.

Precursor lesions showed similar staining changes to those of their normal counterparts after β-elimination. The surface cells decreased in their staining intensity, but other components were unchanged.

PTA: This lectin showed a general decrease in the intensity of its staining of most types

of mucosal cells after β-elimination, especially those in the pit and gland layers. Stromal tissues were not affected by this pre-treatment.

The surface cells in gastritic and metaplastic tissues decreased in their staining intensity, although this change was not striking. Goblet cells were still strongly stained in the metaplastic tissues. Dysplastic lesions also showed a slight decrease in intensity of stain after β-elimination.

MPA: The pre-treatment did not appreciably change the patterns or intensity of staining of normal gastric tissues with MPA. Most cells were well stained as were the stromal tissues.

Some changes were observed in the gastritic and metaplastic tissues, in that the staining intensity was lowered to some extent. Goblet cells were still reactive with this lectin after β-elimination.

DBA: After β-elimination, a lessening of staining intensity was observed in the surface and pit epithelial cells. Gland cells appeared to be unaffected by this treatment. Stroma remained negative in most cases.

Gastritic and metaplastic tissues showed a decrease of staining intensity following β-elimination, with a few exceptions which increased in stain. Goblet cells were unaffected. Dysplastic lesions were also unaffected and remained still strongly stainable.

DSA: The staining pattern of most cells in the mucosal layer remained unchanged. Stroma was still strongly positive. Precursor lesions also remained as they were before β-

elimination.

HPA: HPA yielded a similar pattern of staining of mucosal cells to that before pre-treatment. Neither were changes shown in precancerous lesions.

BPA: A slight diminution of positive staining in normal gastric tissues was observed. There were no other effects.

BSA-II: In normal tissues, this produced the same picture as did direct staining. Gastritic, metaplastic and dysplastic tissues had similar appearances to their normal counterparts as before. The goblet cells in metaplastic tissues remained negative. The overall alterations of staining resulting from pre-treatment by β-elimination, as compared with direct staining were summarised as in table 4.5.

Table 4.5 The results of β-elimination

Lectin	EC	Pit	Gland	Stroma	BV	CG	MP	Dys
UEA-1	=①	=	↓②	↓	↓	↑	=	=
LTA	↑	=	↓↓	=	=	↓	↓G③	↓
PNA	=	=	=	=	=	=	=	=
ECA	=	=	↓	↓	↓	=	=	=
$BSA1B_4$	=	=	↓	=	=	=	=	=
MAA	↑	↑↓	=	=	=	↑↓	↑↓	↑↓
SNA	=	=	=	=	=	=	=	=
VVA	=	=	↓	=	=	=	=	=
WFA	=	↓	↓	=	=	↓	↓	=
SBA	↓	↑	=	=	=	↓	↓	↓
PTA	↓	↓	↓	=	=	↓	↓G	↓
MPA	=	=	=	=	=	↓	↓G	=
DBA	=	=	=	=	=	↓	↓G	=
DSA	=	=	=	=	=	=	=	=
HPA	=	=	=	=	=	=	=	=
BSA-II	=	=	=	=	=	=	=	=

Notes: ①. = means there was not much change of staining intensity and staining patterns in comparison with that of routine staining, ②. ↓ or ↑ means that the staining intensity became decreased or increased after treatment, ③. G refers to goblet cells which were still positively stained.

4.6 LECTIN STAINING OF GASTRIC CARCINOMA TISSUES

The changes of lectin staining patterns in gastric carcinoma different from those of normal, either enhanced or diminished staining, mean that the those glycans detected by lectins have been undergone an alterations, either quantitatively or configurationally or both.

There are generally several possible ways by which the alteration in glycans detected by lectins can occur.

First, the synthesis of mucin core protein is massively increased, which, consequently, provides more sites for the addition of specific glycosyl residues, so that more sugar residues can be incorporated onto the core protein, which leads to the increased expression of a glycan or glycans.

Second, a new core protein, either oncoproteins or mutated forms of normal proteins, is synthesised during gastric carcinogenesis, which will enable the altered glycosylation, leading to the enhanced expression of normal or new types of glycans, so that changed staining patterns can be shown by lectins.

Another possibility is that, since the configuration of oligosaccharide chains is complicated and some glycosyl residues can be blocked by other(s), which makes them undetectable. During oncogenesis and tumour progression, the configuration of one or more types of glycan is changed, resulting in the exposure of the glycans. This may also occur when the degradation of one type of glycan is increased and others are uncovered

as a result.

The majority of lectins stained gastric carcinomata in ways that were different from their normal counterparts, although a minority showed very similar staining intensities and patterns in the normal and pathological tissues. The staining positivity is summarised in table 4.6. The detailed description is as follows.

Table 4.6 The positive staining of lectins on tumours

Lectin	Method	Well-D	Moderately-D	Poorly-D	Diffuse	Signet
PSA	Direct	4/4(100)	5/5(100)	3/5(60)	0/3(0)	2/3(67)
	%	45.3±12.6	28.4±10.5	8.3±4.7	-	38±23
LCA	Direct	2/4(50)	2/5(40)	1/5(20)	1/3(33)	2/3(67)
	%	6.5±4.5	4.5±3.5	4	6	29.5±25.5
e-PHA	Direct	4/4(100)	5/5(100)	4/6(66.7)	3/3(100)	2/3(66.7)
	%	76±11.5	82±12.5	74.4±10.3	82.6±14.5	100
l-PHA	Direct	3/4(75)	4/5(80)	5/5(100)	1/2(50)	2/2100)
	%	24.3±14.2	16.6±11.7	24.9±14.6	48	73.5±21.5
	N'ase	4/4(100)	5/5(100)	4/4(100)	2/2(100)	2/2(100)
	%	56.9±16.5	72.5±13.4	67.8±14.7	82.3±5.3	84.2±10.5
GNA	Direct	2/4(50)	2/5(40)	5/5(100)	2/2(100)	2/2(100)
	%	4.5±4.3	9.5±4.5	13.4±6.4	10.3±4.6	7.5±3.5
NPA	Direct	2/4(50)	2/4(50)	3/5(60)	2/2(100)	1/2(50)
	%	24.5±7.5	5.5±3.5	4.6±3.2	4.2±2.9	4
UEA-1	Direct	3/4(75)	2/4(50)	4/5(80)	2/2(38)	0/2(0)
	%	83±6.6	78.3±11.5	46.3±36.4	15.4±4.3	-
	N'ase	3/4(75)	2/4(50)	4/5(80)	2/3(50)	0/2(0)
	%	79.5±8.5	68.4±12.4	51.4±38.5	14.5±5.5	-
	β-elim	2/4(50)	1/4(25)	3/5(60)	0/2(0)	0/2(0)
	%	18.5	16.5	26.5±11.3	-	-
LTA	Direct	3/4(75)	2/4(50)	4/5(80)	1/2(50)	0/2(0)
	%	72.9±14.2	62.5±15.5	23.8±25.8	9.5	0
	β-elim	2/4(50)	1/4(25)	2/5(40)	0/2(0)	1/2(20)
	%	15.5±6.5	12.5	6.5±2.5	-	61

Table 4.6 The positive staining of lectins on tumours (continued)

PNA	Direct	2/4(50)	2/4(50)	3/5(60)	2/3(67)	1/2(50)
	%	4.5±3.5	6.5±4.5	61.7±2.4	67.5±13.5	36
	N'ase	2/4(50)	3/4(75)	3/5(60)	1/2(50)	1/2(50)
	%	9.5±4.5	7.6±6.8	45.4±19.1	53	62
	β-elim	0/4(0)	2/4(50)	2/5(40)	1/2(50)	1/2(50)
	%	-	8.5±4.5	38.5±26.5	15	16
ECA	Direct	4/4(100)	5/5(100)	6/6(100)	3/3(100)	3/3(100)
	%	81±17.8	65.3±23.4	56.3±14.8	63.1±7.6	75±13.4
	N'ase	4/4(100)	5/5(100)	6/6(100)	3/3(100)	3/3(100)
	%	100	100	100	100	100
	β-elim	4/4(100)	4/5(80)	6/6(100)	3/3(100)	2/2(100)
	%	42.2±8.3	34.3±15.5	60±28.1	78.4±9.6	82±14.3
$BSA1B_4$	Direct	0/4(0)	3/5(60)	2/6(33.3)	1/3(33.3)	0/3(0)
	%	-	3.2±2.2	3.8±1.4	4.2±1.4	-
	β-elim	0/4(0)	2/5(40)	2/6(33.3)	1/3(33.3)	0/3(0)
	%	-	4.1±1.5	3.6±2.1	3.2±2.3	-
SNA	Direct	6/8(75)	6/10(60)	16/19(84)	6/8(75)	3/4(75)
	%	68.6±12.2	55.4±12.4	46.4±22.9	69.2±6.3	70.4±11.3
	N'ase	6/8(75)	5/10(50)	14/19(74)	6/8(75)	3/4(75)
	%	41.3±11.2	28.6±13.5	38±17.9	34.4±8.6	48.6±5.5
	β-elim	7/8(88)	8/10(80)	14/19(74)	6/8(75)	3/4(75)
	%	93±5.9	87.4±6.8	83.8±9.7	71.2±6.5	65.8±18.3
MAA	Direct	9/11(82)	8/10(80)	16/19(84)	7/8(88)	4/5(80)
	%	45.3±23.4	35.6±15.3	36.3±19.8	46.7±19.8	35±18.3
	N'ase	8/11(72)	8/10(80)	14/19(74)	6/8(75)	3/5(60)
	%	42.7±19.7	30.1±8.6	37.3±16.4	38.5±14.5	37.4±10.2
	β-elim	6/8(75)	7/10(70)	15/19(80)	6/8(75)	2/5(40)
	%	58.3±10.2	60.2±5.3	38±27.6	72.3±11.4	5.5±3.7
	N'ase+β-	5/8(63)	7/10(70)	10/14(71)	3/6(50)	2/5(40)
	%	35±10.6	24.5±6.4	40.3±14.3	65.3±8.2	12.3±7.1
LFA	Direct	0/4(0)	3/4(75)	2/4(50)	1/2(50)	0/2(0)
	%	-	60.3±23.2	11.5±4.5	4	-
	β-elim	0/4(0)	2/4(50)	2/4((50)	1/2(50)	0/2(0)
	%	-	28±8.5	9.5±3.5	5	-

Table 4.6 The positive staining of lectins on tumours (continued)

VVA	Direct	2/8(25)	2/10(20)	5/19(26)	2/4(50)	1/5(20)
	%	2.9±2.5	5.1±2.3	22.2±19.1	3.1±1.5	7
	N'ase	0/8(0)	3/10(30)	4/19(21)	1/4(13)	1/5(20)
	%	-	2.2±1.5	6.6±3.2	3	34
	β-elim	0/8(0)	2/10(20)	2/16(13)	0/4(0)	1/5(20)
	%	-	2.3±1.8	3.2±2.4	-	4
WFA	Direct	4/4(100)	4/4(100)	4/5(80)	2/2(100)	2/2(100)
	%	41.2±11.4	56.9±21.3	72.6±11.4	72.5±16.4	79.6±6.8
	N'ase	4/4(100)	4/4(100)	3/5((60)	2/2(100)	2/2(100)
	%	45.9±13.7	56.2±14.3	36.9±21.6	40.5±6.5	74.5±10.5
	β-elim	4/4(100)	4/4(100)	3/5(60)	1/2(50)	2/2(100)
	%	44.3±10.8	34.6±17.6	39.8±17.5	35	69.5±8.5
SBA	Direct	7/10(70)	6/10(60)	9/19(47)	3/6(50)	2/5(40)
	%	15.2±5.4	23.2±9.2	41.2±13.4	23.8±15.2	9.8±5.3
	N'ase	5/10(50)	4/10(40)	7/19(37)	3/6(30)	1/5(20)
	%	20.5±7.4	18.6±4.6	19.5±14.2	11.5±3.8	12
	β-elim	5/10(50)	5/10(50)	6/19(32)	3/6(50)	2/5(40)
	%	21±9.3	16.8±6.7	16.5±13.6	9.6±6.4	12.5±6.5
PTA	Direct	4/4(100)	4/5(80)	3/6(50)	2/3(67)	2/3(67)
	%	81.4±17.8	24.8±16.3	36.4±20.3	32.6±16.2	61±26.9
	β-elim	4/4(100)	4/5(80)	3/6(50)	4/6(67)	2/4(50)
	%	53.2±12.5	16.5±5.3	22.6±3.9	18.2±9.4	25.5±14.5
MPA	Direct	4/4(100)	4/4(100)	5/5(100)	2/2(100)	2/2(100)
	%	68.2±14.7	78.5±12.8	38.4±12.6	69.5±12.5	100
	β-elim	4/4(100)	4/4(100)	5/5(100)	2/2(100)	3/3(100)
	%	67.8±11.7	56.9±18.5	44.3±19.5	65.5±16.5	85.5±14.2
DBA	Direct	2/11(18)	2/10(20)	4/19(21)	2/8(25)	1/5(20)
	%	5.3±2.6	11.5±6.5	14.6±12.1	14.5±6.5	4
	β-elim	0/10(0)	1/10(10)	4/18(0)	2/8(25)	0/5(0)
	%	-	6	9.5±5.4	12.3±1.9	-
STA	Direct	4/4(100)	4/4(100)	5/5(100)	2/2(100)	2/2(100)
	%	68.6±17.6	78.3±14.8	91±7.4	100	70.6±15.6
LEA	Direct	4/4(100)	4/4(100)	5/5(100)	2/2(100)	1/2(50)
	%	78.2±13.6	67.8±15.4	53.2±23.3	71.2±15.4	5

Table 4.6 The positive staining of lectins on tumours (continued)

DSA	Direct	4/4(100)	5/5(100)	6/6(100)	2/2(100)	2/2(100)
	%	68.5±20.4	66.5±21.2	45.6±29.3	76.5±15.5	82.5±16.5
	N'ase	4/4(100)	5/5(100)	6/6(100)	3/3(100)	3/3(100)
	%	100	100	100	100	100
	β-elim	4/4(100)	5/5(100)	6/6(100)	3/3(100)	3/3(100)
	%	70±12.5	76.3±10.6	76.4±17.5	100	100
HPA	Direct	4/4(100)	5/5(100)	4/6(67)	2/2(100)	3/3(100)
	%	65.3±21.6	59.4±18.5	45.6±24.5	61.5±20.5	32.4±14.6
	N'ase	4/4(100)	4/5(80)	5/6(83)	3/3(100)	3/3(100)
	%	70.5±16.4	60.5±15.7	54.2±19.5	20.6±13.8	76.2±12.5
	β-elim	3/4(75)	4/5(80)	4/6(67)	2/3(67)	3/3(100)
	%	25.6±12.3	32.5±19.5	25.6±13.5	19.8±3.5	65.6±17.4
BPA	Direct	3/4(75)	2/4(50)	4/5(80)	2/2(100)	2/2(100)
	%	16.3±10.2	34.5±10.5	45.3±16.4	40.5±15.5	43.5±17.3
CTA	Direct	2/4(50)	1/4(25)	4/6(67)	2/2(100)	2/2(100)
	%	6.5±4.5	4	45.3±19.2	29.6±14.5	21.5±11.5
BSAII	Direct	2/4(50)	2/4(50)	4/6(67)	2/2(100)	1/2(50)
	%	15±7.5	12.5±6.5	19.4±7.4	9.6±4.6	11
	β-elim	1/4(25)	1/4(25)	3/6(50)	1/2(50)	1/2(50)
	%	8	8	12.6±7.2	9	5

PSA: Generally, all types of tumour tissues were focally stained, in which a small proportion of tumour cells showed positive staining both cytoplasmically and on nuclear membranes. Well-differentiated gastric carcinomata displayed a comparatively higher proportion of positive tumour cells (Figure 4) than the other types of carcinomata. The positive cells showed perinuclear cytoplasmic granules with clear membranous staining. Moderately-differentiated carcinomata showed a decreased proportion of positive cells (28%), while poorly-differentiated tumours showed only about 10% of stained cells. Variations were observed in that some tumours did not bind to this lectin. Diffuse type

carcinomata were generally not stained and signet ring cell tumours appeared to have a higher percentage of PSA-reactive cells (Figure 5). Compared with the normal counterparts, the staining patterns of tumour cells resembled those of glandular cells.

LCA: This lectin produced a similar staining pattern to that of PSA. Well-differentiated carcinomata had a low ratio of positively stained cells and some were even negative (Figure 7), the moderately-differentiated type of gastric carcinomata also had a low ratio of positive cells (5%) and the poorly-differentiated type was rarely stained in most cases

(4% in one case). The signet ring cell type of carcinoma displayed more positive cells (up to 55%). Again, this staining pattern was similar to that of normal gland cells.

e-PHA: This lectin stained the lumenal parts of the gastric tissues and it gave no significant differences between tumours and their normal counterparts or among the different types of tumours. It was noticed that some variations of staining intensity occurred in poorly-differentiated gastric carcinomata, in which some tumour cells were not stained by this lectin. The cytoplasm of tumour cells was rarely stained (Figure 9).

l-PHA: This lectin gave different staining patterns from PSA and LCA on the intestinal type of gastric carcinoma. Well-differentiated tumours had a low proportion of positive cells (24%), which showed lumenal membrane and intercellular staining (Figure 11), while poorly-differentiated tumours yielded a similar proportion of lumenal membrane-positive cells, although variations of the number of positive cells occurred between cases. The simple diffuse type of gastric carcinoma had about half of the tumour cells stained. Signet ring-cell tumours, on the other hand, showed cytoplasmic positivity in most

tumour cells (74%) (Figure 12). This phenomenon was much like the staining of goblet cells in metaplastic tissues, in which goblet cells were strongly stained by l-PHA.

Neuraminidase digestion dramatically increased the 'positivity rate' of normal mucosal cells and changed the staining intensity of almost every type of gastric tumour. In well-differentiated tumours, the positive cell percentage increased and nearly all cells showed positive staining of the lumenal membrane rather than cytoplasm. About 68% of tumour cells in the poorly-differentiated and 82% in diffuse types of tumours showed positive staining, mainly on their membranes. In the signet ring cell tumour, all cells were stained cytoplasmically.

GNA: A low percentage of positive tumour cells was observed in well-differentiated carcinomata and some did not bind this lectin at all (Figure 14). Increased and scattered staining was seen as much as 16% of cells in the poorly-differentiated type, 10% in the diffuse type and 5% in the signet ring cell type. It appeared that these staining patterns were similar to that of normal mucosal surface epithelial cells, which showed a few positive cells here and there.

NPA: A very few positive cells were thinly distributed in all types of gastric carcinomata, as shown in Figure 16. Most tumours produced about 5 percent of positively stained cells, except for well-differentiated carcinomata, which had about 25% of cells with positive lumenal membranes and intercellular junctions.

UEA-1: This lectin showed variations of staining patterns among different types of tumours. In high grade gastric carcinomata, positive staining was observed in all tumour

cells on the lumenal surface (Figure 20). Lower grade tumours showed a high degree of heterogeneity in staining of tumour cells. This was especially evident in poorly-differentiated carcinomata, but some still showed high numbers of positive cells (Figure 21). Carcinomata of the diffuse type had a low positive percentage of tumour cells. Signet ring cell carcinomata were completely negative.

Neuraminidase digestion did not change these staining patterns in most cases, except for tumours of the diffuse type in which the positive cell count dropped to zero.

Pre-treatment by β-elimination, on the other hand, lowered the proportion of tumour cells stained in most cases. The percentage dropped from about 83% to 19% in well-differentiated tumours and from as high as 78% to 17% in the poorly-differentiated type. Diffuse carcinomata completely lost their staining.

LTA: Different types of gastric carcinomata showed different and distinct staining patterns with this lectin. Well-differentiated tumours were reactive to the lectin and nearly all tumour cells were positively stained on their lumenal membrane (Figure 24), and like the staining of UEA-1. Poorly-differentiated tumours had more heterogeneous staining than high grade ones and generally showed about 23% of positive cells, with a few cases showing a high percentage of positive staining (70%). Diffuse type and signet ring cell carcinomata were unstained.

Beta-elimination changed these staining patterns. Well-differentiated tumours showed decreased staining intensity and decreased proportion of positive cells. Tumours of the poorly-differentiated type also showed a sharp reduction in membrane staining, which led

to a total loss of staining in one case. The simple diffuse type of carcinoma became negative. Signet ring cell carcinomata, in contrast, showed up to 61 percent of 'cytoplasm-positive' tumour cells. This phenomenon is consistent with the changes of staining patterns of normal mucosal cells, in which the surface and pit cells became positively stained only after β-elimination.

PNA: PNA stained all mucosal epithelial cells. It also showed widespread staining of the cells of most types of carcinomata, except for the well- and moderately-differentiated tumours. High-grade tumours displayed a low percentage of positive cells (5% and 7% in well- and moderately-differentiated tumours, respectively), but more cells were stained in adjacent areas. Low-grade tumours generally exhibited a high proportion of positive tumour cells. Poorly-differentiated carcinomata showed about 62% of PNA-reactive cells with cytoplasmic staining, simple diffuse tumours had 68% of positive cells, while signet ring cell tumours had only 36% of positively stained cells.

Neuraminidase digestion did not significantly alter the staining of tumour cells. The high-grade tumours still showed low proportions of positive cells and low-grade tumours had much high percentages of positive cells, but the proportions of positive cells dropped significantly, in comparison with direct staining, in poorly-differentiated neoplasms. The diffuse type of carcinomata tended to show more positive tumour cells.

Beta-elimination, in contrast, strikingly decreased the number of positive cells in most gastric neoplasms. Well-differentiated tumours lost all their staining, poorly-differentiated carcinomata had 39% of PNA reactive cells and one case lost staining entirely. Diffuse tumours decreased from 68% to 15% of stained tumour cells and one

case lost staining. Signet ring cell tumours also lost part of their positive cell population, which fell to 16%.

ECA: This lectin stained most components of normal gastric mucosa intensely and most tumours showed high percentages of positive cells. These ranged from 56% in poorly-differentiated tumours, with similar ratios in simple diffuse and signet ring cell tumours, to 81% in the well-differentiated tumours. Intramucosal tumour cells were intensely stained on their lateral aspects (Figure 30). In the adjacent area, it was noticed that the tumour cells were stained as strongly as adjacent remaining gland cells (Figure 31), the latter were usually not intensely staining in normal tissues.

Neuraminidase treatment increased the number of positive cells to nearly 100 percent, with cytoplasmic staining. Beta-elimination decreased the staining intensity and proportion of tumour cells in the intestinal type of gastric carcinomata, while the percentage of positive cells in diffuse-type carcinomata remained at about the previous levels or were slightly decreased.

BSA1-B_4: All types of gastric carcinomata showed very low percentages of positively stained cells, which were generally less than 5%. In well-differentiated tumours, positive cells were scattered along the granular tubules and the stained parts were mainly on the lumenal membrane, while cytoplasmic staining was faint. A similar staining was shown among the poorly-differentiated carcinomata, which had an average of 4% of stained cells and 4 out of 6 tumours were negative. The staining of simple diffuse tumours was almost the same as that of the poorly-differentiated carcinomata with a very low proportion of positively stained cells. Signet ring cell tumours were negative.

Beta-elimination led to the disappearance of the previous staining in high-grade tumours, but it did not change the staining of low-grade types of tumours. Signet ring cell tumours remained unstained.

SNA: This lectin stained a high proportion of tumour cells apically, rather than cytoplasmically, in well-differentiated carcinomata (Figure 35). The number of positive cells varied in poorly-differentiated tumours, ranging from 25% to 65% with an average of 46%, the stained areas were mainly on the lumenal side. The diffuse and signet ring types of carcinomata had about 70% of positive cells.

Neuraminidase digestion decreased the fraction of positive cells in most tumours: about one-third of previously stained cells in signet ring cell tumours lost their staining. Half of the cells stainable before treatment lost their staining in poorly- and moderately-differentiated tumours.

Beta-elimination tended slightly to increase the number of positive cells in the intestinal-type carcinomata. Nearly all (93%) of well-differentiated tumour cells showed as 'membrane positive', 87% in moderately-differentiated and 83% in poorly-differentiated tumours. Simple diffuse and signet ring cell tumours did not change.

MAA: In the well-differentiated type of gastric carcinoma (Figure 37), the positive cells accounted for an average of 45% of the total tumour cell population and usually showed lumenal membrane staining. In the moderately-differentiated type, the fraction of positive cells dropped to about 36%. In the poorly-differentiated type (Figure 38), the fraction of

positive cells varied widely from 11% to 80% with a mean value of 36%. Carcinomata of the diffuse type had 47% of positive cells and in signet ring cell tumours, most cells were positive cytoplasmically (Figure 39).

Neuraminidase digestion tended slightly to decrease the proportion of tumour cells stainable with MAA, especially in the diffuse types of carcinomata. The other types of gastric carcinoma retained at about the same proportions of 'positive' cells as before enzyme treatment.

Beta-elimination tended to enhance the staining intensity of tumour cells. It increased both staining intensity and proportion of cells stained in high-grade tumours, but not in low-grade tumours. The staining of the poorly-differentiated tumours remained at about the same level as that without treatment. The same applied in the diffuse type. Staining in the signet ring cell carcinomata, on the other hand, was strikingly diminished by this treatment and most cells lost their staining.

The effects of neuraminidase digestion and β-elimination together had generally similar effects on the staining of tumour tissues to that of neuraminidase digestion alone, though certain differences were noteworthy. Nearly all tumour tissues were observed to decrease in both staining intensity and in the proportion of positive cells. This tendency was much more obvious in the signet ring cell tumours, in which only 12% of tumour cells showed positivity.

LFA: LFA stained a low proportion of tumour cells in poorly-differentiated and diffuse gastric carcinomata, but a higher proportion in tumours with moderate differentiation

(60%) (Figure 41). There was no staining in the well-differentiated and signet ring cell tumours. Staining was also found in the metastatic cells in lymph nodes, which showed membranous staining. Beta-elimination had no effect on staining with this lectin.

VVA: Most tumour cells were unstained in the majority of the specimens. It was noticed that intramucosal tumour cells showed membrane staining (Figure 45). Only a very small proportion of positive tumour cells was detectable, and was scattered, in well-differentiated carcinomata. In signet ring cell tumours, one case was stained with 7% of tumour cells. Positive cells were seen on the lumenal membrane in poorly-differentiated carcinomata (Figure 46).

After neuraminidase treatment, the proportion of stained tumour cells was slightly decreased. Fewer than 5% of cells were stained in high-grade carcinomata. Notably, 34% of positive cells were positive in signet ring cell neoplasms.

Beta-elimination reduced the number of positive tumour cells almost to zero in most specimens. Only few stained cells were seen, scattered individually or in tiny clusters.

WFA: Normal gastric mucosal cells were strongly stained by this lectin, as were the tumour cells, as shown in Figures 48 and 49. Increased proportions of positive cells were observed in the poorly-differentiated, diffuse and signet ring cell carcinomata.

As on the normal mucosa, neuraminidase digestion decreased the staining intensity of tumour epithelium in low-grade carcinomata, though to varying degrees. Well- and moderately- differentiated tumours showed a slight increase in the proportion of

'positive' cells with lumenal membrane staining. An obvious heterogeneity was seen in poorly-differentiated carcinomata, which yielded 'positive' cell percentages ranging from as low as 12% to as high as 71% with an average of 37%. In the diffuse carcinomata the positive population was also diminished to about 41%. Signet ring cell tumours were less affected, retaining about 76% of positive cells.

Beta-elimination , in general, lowered the staining intensities from +++ to ++. The percentage of positive cells markedly decreased in the moderately-, poorly- differentiated and diffuse carcinomata, but no change was evident in the well-differentiated and signet ring cell carcinomata.

SBA: With the standard staining procedures, well-differentiated tumours tended to have fewer cells positively stained (Figure 52) than did other types of gastric carcinomata. The poorly-differentiated carcinomata gave a higher proportion of positive cells than other types (Figure 53). Signet ring cell tumours had the lowest proportion of stainable tumour cells (10%).

After neuraminidase digestion, the numbers of 'positive' tumour cells in well-differentiated gastric carcinomata increased slightly, but most poorly-differentiated and diffuse carcinomata lost part of their staining cell population and had clustered positive cells. Signet ring cell tumours showed no change after this treatment and remained at about 12% of positive cells.

After β-elimination, most tumour cells were no longer reactive to SBA, leaving a very few positive cells dotted sparsely. Well-differentiated tumours showed positive cellular

staining limited to the surface layer. Most other type of carcinomata generally lost part of their staining population. Signet ring cell tumours were unchanged.

PTA: The 'positive' cell fraction was very high in well-differentiated carcinomata (81%) (Figure 56) and in signet ring cell tumours (61%) (Figure 57). All other types of tumours showed low percentages (as 25%, 36% and 33% in moderately-, poorly-differentiated, and diffuse carcinomata, respectively).

Beta-elimination decreased the fraction of positive cells in most cases. This phenomenon was consistent with the effects on normal mucosal cells, which also showed general lessening of staining intensity and positive cell fraction after β-elimination.

MPA: As described above, this lectin stained almost every component in the normal mucosa intensely. It also stained a high proportion of tumour cells, though variations were observed between different types of tumours. Most well-differentiated carcinomata were stained on surface membranes (Figure 61), with nearly all the tumour cells being membrane-positive. Poorly-differentiated tumours showed between 30% and 60% of 'positive' cells while the diffuse type had up to 70%. Nearly all cells had cytoplasmic staining in the signet ring cell carcinomata.

Beta-elimination raised the fraction of cells stained in poorly-differentiated carcinomata from 45% to 60%. In other types of tumour the percentages of positive cells were unchanged.

DBA: DBA did not show binding to most tumour cells. It was observed that DBA

hardly stained carcinoma cells in well- and moderately-differentiated tumours: only a few positive cells were scattered in the tissues. Poorly-differentiated tumours had very similar staining. Signet ring cell tumours also showed a very low proportion of signet ring cells with cytoplasmic positivity.

Beta-elimination severely decreased the previous staining of the positive cases, most cases of which became negative after this treatment. A few specimens still showed scattered positivity.

STA: Most of the tumour cells were as strongly stained as their normal counterparts, with staining either on the lumenal surface in well- and poorly-differentiated types (Figure 68) or in cytoplasm (signet ring cell type) or both (in the diffuse type).

LEA: Generally, the majority of tumour cells were stained in all types of gastric carcinomata. The positive percentages tended to decrease from high-grade tumours to low-grade tumours, such as 78% in well-differentiated carcinomata to 53% in poorly-differentiated carcinomata (Figure 70), and comparatively higher numbers of positive cells were observed in the diffuse type of tumours (71%), all with lumenal membrane staining rather than cytoplasmic granules. Exceptionally, signet ring cell tumours were nearly negative, with a few positive cells scattered in the tissues.

DSA: In well-differentiated gastric carcinomata, generally most of the tumour cells were stained intensely in the cytoplasm with clear membrane outlines (69%). The set of poorly-differentiated carcinomata showed apparent heterogeneity, and the positive rates were between 40% and 80% among cases with strong lumenal staining. Tumours of the

diffuse type also had up to 77% of stained cells. Signet ring cell tumours showed about 83% of DSA-reactive cells.

Neuraminidase pre-treatment increased staining of tumour cells and nearly all cells were stained on their membranes and/or cytoplasmically. Beta-elimination had no obvious effect on the staining patterns or their intensity.

HPA: Most tumours reacted positively with high proportions of stained cells. In well-differentiated tumours, the positive cells accounted for about 65% of the total, with clear apical membrane staining (Figure 77). Different 'positive' rates from as low as 16% to as high as 80% were seen in the poorly-differentiated carcinomata with an average of 46% of positive cells. The diffuse-type tumours also showed variable proportions of HPA-reactive cells (Figure 78), while signet ring cell tumours showed about 32% of cytoplasmic and membrane-positive cells.

After neuraminidase digestion, the overall staining was enhanced in most tumour tissues. Well-differentiated tumours remained at about the same positive fraction as before. Poorly-differentiated carcinomata slightly increased their numbers of HPA-reactive cells, leading to 54% of positive cells. The proportion of positive cells was significantly decreased in diffuse carcinomata. In the signet ring cell tumours, more tumour cells (76%) were positively stained after this treatment.

Beta-elimination tended to lower the 'positive' proportion of tumour cells and led to a sharp decrease in the numbers of stained cells in well- and poorly-differentiated and diffuse-type carcinomata. Signet ring cell tumours seemed to be largely unaffected by this procedure.

BPA: In general, this lectin reacted with small proportions of tumour cells and the staining tended to be weak. Tumours with high differentiation showed no positive cells than did the moderately- and poorly-differentiated carcinomata, though some variations were seen among individual cases (Figure 81).

Neither neuraminidase digestion nor β-elimination yielded any changes in the staining of the tumours.

CTA: This lectin produced very variable and weak staining among the different types of carcinomata and within each type of tumour. The 'positive' cell fraction varied: 6% in well-differentiated tumours and from negative to 67% in the poorly-differentiated type. Diffuse tumours had about 30% of positive cells and signet ring cell carcinomata had an average of 21% with cytoplasmic staining (Figure 83).

BSA-II: Most specimens showed very low proportions of 'positive' cells. In well-differentiated tumours, such positive tumour cells accounted for 15% of the total cell population. Similar percentages were found in the poorly-differentiated and diffuse types of carcinomata. Signet ring cell tumours were unstained by this lectin.

After β-elimination, the 'positive' cell population generally decreased and some poorly-differentiated tumours lost their staining.

4.7 SIALYL LEWIS^A STAINING ON VARIOUS GASTRIC TISSUES

In normal gastric tissues this antigen was rarely expressed on epithelial cells of the gastric mucosa (Figure 86). The majority of surface epithelial cells did not show staining, except

that a small proportion of these cells showed faint cytoplasmic stain in a number of 'positive' cases. A few scattered gland cells had cytoplasmic stain.

In the gastritic tissues, some of the epithelial cells showed variation in their staining and some showed increased positivity, especially the cells in areas with active proliferation. Metaplastic tissues generally showed an increased staining (Figure 87). The goblet cells in the metaplastic tissues showed variations in their staining, some were cytoplasmically positive and some were negative. Dysplastic tissues displayed an enhanced cytoplasmic staining.

Gastric carcinomata expressed this antigen in various proportions of tumour cells. The secretions of tumour cells showed a very strong staining in most tissues, as detailed below.

In well-differentiated tumours, the positively stained parts were mainly the membrane of the cells (Figure 88), though some cells were cytoplasmically positive. Variations of staining were observed among tumour cells; some cells in the same glandular structure were positive while others were unstained. A similar phenomenon occurred at a large scale among glandular structures, parts were positive and others were negative.

The staining of the moderately-differentiated carcinomata resembled the staining patterns of the well-differentiated tumours. There was a slight difference between them in the proportion of 'positive' cells, especially those with cytoplasmic stain. In moderately-differentiated tumours the percentage of cells stained (68%) was higher than in well-differentiated carcinomata (43%).

The staining in poorly-differentiated tumours showed a high degree of heterogeneity. Most tumour tissues was positively stained and the proportions of positive cells differed markedly from one tumour to another (25%-78%). The membrane was still the main positively stained part, but cytoplasmic staining was common. The staining in the invasive boundaries was more easily seen than in the centre of the cell masses.

The diffuse type carcinomata also showed a variable heterogeneous staining among specimens (Figure 89). Some cases did not express the antigen and some showed variable percentages (from 28% to 69%) of membrane and /or cytoplasmic positive tumour cells. Signet ring cell carcinomata showed a comparatively high proportion (up to 74%) of Lewis^a-positive cells.

4.8 MUC1 STAINING ON GASTRIC TISSUES

The staining for MUC1 was generally negative in the normal gastric mucosal cells, and a few specimens showed low percentages of stained cells in glands (Figure 90). In some of the gastritic and metaplastic tissues, positive staining was shown sparsely scattered in a few affected cells. Dysplasia produced rare positive staining in a few proliferative cells, but most dysplastic cells did not stain for MUC1.

Tumour tissues again showed heterogeneity in both their staining patterns and intensity. Four out of 11 cases of well-differentiated gastric carcinomata exhibited scattered and heterogeneous 'positive' tumour cells in glandular structures (Figure 91), while the majority of tumour cells remained unstained. It appeared that more cases (6 out of 10) of moderately-differentiated carcinomata showed positive staining mainly on the lumenal surface of tumour cells. More heterogeneous staining was seen in poorly-differentiated

and diffuse-type carcinomata, which showed percentages of positively stained tumour cells from as low as 5% to as high as 85% (Figure 93).

The detail of Lewis^a and MUC1 staining in gastric tumours was summarised in table 4.7.

Table 4.7 The expression of Lewis^a and MUC1

	Well	Moderately	Poorly	Diffuse	Signet ring
Lewis^a	7/11	5/10	12/19	4/7	3/5
	43.2±15.4	67.8±16.3	55.7±20.3	44.6±16.2	52±14.6
MUC1	4/11	6/10	9/19	4/8	2/5
	15.2±5.4	11.3±4.4	43.6±35.2	30.5±23.3	3.4±2.5

CHAPTER 5 DISCUSSION

5.1 GENERAL OVERVIEW

Lectins were first discovered over a hundred years ago. Since then the number known has expanded enormously, especially in recent several decades, and a wide range of labelled lectins, with a variety of sugar specificity, are commercially available. Hence, the histological exploration of those molecules that contain carbohydrates, such as mucins and other glycans in the gastrointestinal tract, can now be pursued with a previous unattainable degree of chemical details. Besides, the applications of monoclonal antibodies against glycans also enhance the studies on the glycans, so do the glycosidase.

Lectins was first applied to the gastric mucins in the early 1970's (Lotan and Sharon, 1978) and at that time, rather few lectins were commercially available. Subsequently studies of lectin-binding gastric mucosae were made on both animals and human beings. A modest number of lectins have since been employed on those tissues which were either normal or showed various gastric pathologies. These studies have yielded promising results and consequently more attention has recently been attracted to this field and especially on the neoplastic diseases.

Those lectins which have previously been applied to the study of gastric mucins and other gastric glycoconjugates have shown their potential usefulness, but because of the limited number of lectins used and the somewhat unsatisfactory choices of lectins sometimes made, these studies have only generated a modest amount of biochemical information either about normal glycoconjugates or their pathological alteration.

Therefore, there was a need to obtain a wider overview of the structures of gastric glycans in normal and pathological states, by using a more carefully selected range of lectins and other probes. From such an overview chemical information could be derived that would guide further biochemical studies.

A total 27 lectins were used in the study. Among them, 13 have been used in previous histochemical analyses of gastric tissues as summarised in chapter 2 (for references see chapter 2). Another 14 lectins are here being used for the first time in studies of the glycan in gastric tissues.

These lectins cover all four types of glycans. There are several lectins chosen to explore the same glycan with subtle differences in sugar sequences, for example, SNA and MAA are both for sialyl glycans, but SNA is for galactosamine with α2,6 linkage to neuraminic acid, which MAA is specific for α2,3 linkage.

5.2 STAINING PATTERNS OF DIFFERENT GASTRIC MUCOSA

As was summarised in chapter 4, some lectins stained gastric mucosae very intensely, some showed preferential staining of particular cells and some stained none of them. Generally, four lectin staining patterns were found among normal gastric mucosal cells.

5.2.1 All mucosal cells stained

A number of lectins stained all kinds of mucosal cells, of both epithelial cells and glandular epithelium, very intensely, which implies that certain glycans were abundant in all types of gastric mucosal cells. The sugar groups revealed by these lectins can be

tentatively identified as: **e-PHA** (Bi/tri antennary, bisected complex N-linked sequences); **l-PHA** (Tri/tetra antennary, bisected complex N-linked sequences); **PNA** (Galβ1,3 GalNAc α1> Gal β1,4 GlcNAc β1); **ECA** (Gal β1,4 GlcNAc β1-); **WFA** (GalNAc α1,6 Gal β1->GalNAc α1,3 Gal β1-); **MPA** (Gal β1,3 GalNAc α1-); **STA** [(-4 D-GalNAc $\beta 1\text{-})_2$ > 3 Gal β1,4 GlcNAc β1-]; **LEA** [$(\text{-4 GlcNAc } \beta 1\text{-})_2$ >> Gal β1,4 GlcNAc β1-]. Some of them were also strongly expressed in stromal tissues including e-PHA, ECA, MPA and STA. PNA is an exception, which did not show any staining in stroma.

5.2.1.1 e-PHA

e-PHA stained every component of the gastric mucosa strongly, suggesting the abundance of bi/tri antennary, bisected complex N-linked sequences. This also applied in the precancerous lesions including chronic gastritic, metaplastic and dysplastic mucosa, as well as in all types of gastric carcinoma tissues. That is, the sugar sequences detected by e-PHA seems not readily affected by various pathological conditions, suggesting that there is no significant genetic and/or biochemical modifications arising from the action of diverse pathogenic factors.

5.2.1.2 l-PHA

Although l-PHA stained mucosal cells, its intensity was much lower than that exhibited by any other lectin in this category, which was recognisably positive. Part of surface cells did not show detectable staining in some cases. Neuraminidase digestion did not change the overall staining patterns in most cases, implying that the l-PHA-specific glycosyl sequences are not, at least directly, linked to neuraminic acids, or alternatively, not covered by neuraminic acids.

The precancerous lesions showed small variations, with either an increase or a decrease in staining intensity. Neuraminidase pre-treatment tended to diminish the staining intensity of these lesions.

The proportion of cells showing staining and the stain intensity in most types of gastric carcinoma cells were markedly reduced. Only a very low percentage (<25%) of the intestinal type of gastric tumours were stained, but there was a tendency for the proportion of cells stained to increase in this type of tumour. In the diffuse carcinomata, over 48% of the tumour cells were positive.

This might imply that there is a possible change of the glycan synthetic process in different types of gastric carcinomata during the tumour occurrence and progression. Glycan synthesis may be suppressed during carcinogenesis, which led to the sharp reduction of the sugar sequences for l-PHA. During tumour progression, the synthesis for l-PHA specific sequences gained certain recovery, so that more tumour cells expressed these sequences in the low grade carcinoma such as in poorly-differentiated and diffuse carcinomata.

5.2.1.3 PNA

PNA tended to show differential staining between the parietal cells and the chief cells, in that the former were less intensely stained than the latter. Macartney *et al* (1986) reported results similar to those above. They found that PNA bound strongly to all kinds of cells in the gastric mucosa. However, some other authors have reported different findings (Fisher *et al*, 1983a).

Fisher *et al* stated that there was no staining of PNA on the surface epithelial cells of gastric mucosa, but this lectin did stain the rest of the mucosal components. Peschke *et al* (1983) found that PNA bound widely to different gastric mucosal cells. In their report, PNA produced a mixed outcome with almost all mucosal cells. They suggested that different carbohydrate moieties, mainly in the terminal position occurred in morphologically identical cell types. Macartney *et al* (1987) gave some support to the observations above. It is unclear what the cause of these differences between authors is, but a probable cause is the use of the different lectin concentrations, coupled with other minor variations of techniques.

Neuraminidase digestion slightly reduced the staining intensity of mucosal epithelium, but β-elimination appeared to have no effect on the intensity of staining. This indicates that part of the sugar sequences detected by PNA have close to, or exist in the salic acid complex, as staining was diminished by neuraminidase digestion, and mainly N-linked to the peptide bone, as unaffected by β-elimination.

PNA binding showed an overall decrease in all three types of precancerous lesions, especially in the metaplasia, suggesting that the synthesis of PNA-specific sequences is sensitive to the cellular changes induced by inflammatory factors. In gastric carcinomata, significant differences were observed among different types of carcinomata. Highly differentiated tumours (i.e. in the well- and moderately-differentiated groups) showed a very low proportion of positively stained tumour cells (3.4% and 6.2%, respectively). In contrast, over one third of poorly differentiated tumour cells showed a positive stain with PNA.

The results may indicate that changes of the glycans for PNA occur in the early stage of gastric carcinogenesis, reflecting in the decrease of staining intensity in precancerous lesions. These changes continued and became severely impaired as shown in high grade of gastric tumours which showed low proportions of positive tumour cells. The synthetic process might be recovered with the tumour progression, as shown in low grade of tumours which gave a high proportion of stained cells.

β-elimination decreased the proportions of carcinoma cells staining in most cases, suggesting that there might a transition of N-linked glycans to O-linked dominance or PNA-specific sequences appear more in O-linked oligosaccharides. The mechanisms need to be clarified.

5.2.1.4 ECA

This lectin stained all type cells in the gastric mucosa strongly. Slight variations were observed in the gland layer, in which some cells did not showed staining. Neuraminidase digestion diminished the staining intensity of surface and pit cells, but not of the gland cells and other mucosal components. β-elimination was of no effect on the mucosal cells.

Chronic gastritis, metaplasia and dysplasia all showed slight diminution in staining with ECA relative to normal mucosa. Neuraminidase digestion and β-elimination had no effect. Gastric carcinomata were generally decreased relative to normal and preneoplastic lesions, because of a fall in the fraction of cells stained (i.e. 91% of positive in the well-differentiated carcinomas and about 63% to 75% in other types of gastric tumours). Neuraminidase digestion increased the level of staining in all tumour cells. This is probably due to the removal of sialic acid residues which may occlude the ECA-binding

sequences. The decrease in staining by β-elimination could indicate the increase of O-linked glycans in tumour cells which are susceptible to this treatment, suggesting a possible synthetic alteration occurs in the gastric carcinogenesis and progression.

5.2.1.5 WFA

This lectin binds to GalNAc α1,6 Gal β1- with an affinity about fifty times greater than that it shows to GalNAc α1,3 Gal β1-. Its ligand seemed to be very commonly expressed in the mucosal cells, since the staining with WFA showed a uniformly clear pattern in all the gastric mucosal epithelial cells as well as cells of glands. Some variations in the gland cells were noticed, in that the parietal cells appeared to stain slightly more strongly than chief cells. WFA did not staining stromal tissue and produced a clear contrast between the epithelial cells and the stroma, implying a differential synthesis of the WFA ligand in the epithelial cells.

Pretreatment with neuraminidase and β-elimination slightly decreased the reactivity towards WFA, suggesting that WFA ligand occurs on both O- and N- glycans and that at least part of it is associated with salic acid, though probably indirectly.

None of the precancerous lesions showed evident changes from normal in their staining patterns and intensity of stain with WFA. Hence, the synthesis of WFA ligand was not greatly affected by the inflammatory and other reactive process occurring. There was, likewise, no change in the effects of neuraminidase and β-elimination.

The majority of neoplastic cells in all the types of gastric carcinomata showed positive

staining with WFA. Neuraminidase digestion reduced the percentage of positively stained tumour cells in most cases. β-elimination also diminished the staining intensity and the number of tumour cells stained with WFA. This implies that more O-linked glycans containing GalNAc α1,6 or α1,3 Gal β1- sequences are expressed as the tumours progress.

In summary, WFA is an indicator for differentiating cells of epithelial origins from those of mesenchymal derivation and can be used to evaluate the increased expression of O-linked glycans as the tumours progress.

5.2.1.6 MPA

MPA is a lectin less studied in gastric tissues than has PNA (Okamoto and Forte, 1988). In their study attention was focused on the distribution of its ligand on gastric surface mucosal cells of the rat. It was shown that this lectin had bound strongly to the surface cells and stained mainly in the rough endoplasmic reticulum (i.e. the cytoplasm).

The study described here (see chapter 4) showed a similar pattern, but in man, in that mucosal cells were generally cytoplasmically stained by MPA.

MPA binds with high affinity to Gal β1,3 GalNAc α1 sequence-specific (Lotan and Sharon, 1978; Sarkar *et al*, 1981), with which it interacts via two subsites within its binding site, and with much lower affinity to GalNAc α1- with which only one subsite interacts. It has, therefore, a similarity in high affinity binding to PNA, but the lectins differ in that PNA can also bind to Gal ß1,4 GlcNAc β1- sequence, unlike MPA; while

MPA binds to GalNAc α1- sequence, unlike PNA (Sueyoshi *et al*, 1988). Hence, MPA can give a different staining patterns from that produced by PNA, depending upon the sequences present and their abundance and density.

MPA did not show any preferential staining in precursor lesions and in most cases the staining remained as strong as in normal mucosa. Enzymatic and chemical modifications did not bring about any changes of staining.

In gastric carcinoma tissues, a decrease in staining with MPA was observed, but only in poorly-differentiated and diffuse types of tumours, which showed 48% and 69% of positive tumour cells, respectively. Other types of carcinomata showed no changes from normal. These results differ from those obtained with PNA, probably because of their differential specificities (see above). The further resolution requires more extensive enzymatic degradation and chemical analysis.

5.2.1.7 STA

STA did not yield any preferential staining both in normal and tumour cells, since staining was intense in both cases. Some reduction of stain in precancerous conditions were noticed, especially in metaplastic and dysplastic lesions. The reasons for this are unclear. It may result from the biochemical changes consequent upon the inflammation, but no further substantial modifications were detected in the synthetic process during tumour progression.

5.2.1.8 LEA

The patterns and intensity of stain given by LEA were similar to those of l-PHA, in that

LEA weakly stained the epithelial cells, but it showed a variable staining in the gland cell layer. It also produced a weak staining in the stromal cells. Less abundance of the glycosyl sequences containing (-4 GlcNAc β1-)$_2$ or Gal β1,4 GlcNAc β1- sequences were, therefore, only sparsely present.

A general decrease in staining intensity was seen in gastritis, metaplasia and dysplasia. The same phenomenon was also observed in the staining of the tumour cell, although the decrease was much less obvious. However, a decrease in the fraction of tumour cells stained was shown in signet ring cell carcinomata. This may mean that there is a change in the glycosyl transferase activities which synthesise glycans containing (-4 GlcNAc β1-)$_2$ or Gal β1,4 GlcNAc β1- sequences, either at the genetic level, or by a failure of the reactants to approximate, or by way of biochemical regulation. Alternatively, there may be a failure to synthesise or glycosylate a protein (or proteins) being the ligand glycans. LEA may be of use as a differentiating factor in tumour subtyping.

5.2.2 Surface and pit cells

Some lectins stained sugar residues preferentially in epithelial cells, but stain was diminished or absent in gland cells. These were interpreted as probably arising from the following glycans: **BSA1-B4** (Gal α1,3 Gal β1,4 GlcNAc ß1-); **VVA** (GalNAc a1,3 Gal β1-); **SBA** (terminal GalNAc a1,3- > Gal a1-); **DBA** [GalNAc α1,3(Fuc α1,2) Gal β1,3/4 GlcNAc β1-] and **CTA** (Gal β1,4 GlcNAc- in certain branching patterns).

5.2.2.1 BSA1-B$_4$

This lectin showed a very clear staining in both surface epithelial and pit cell layers, in

which all cells were strongly cytoplasmically stained, while other components of the gastric mucosa were completely negative. Thus a bright contrast was produced. This suggests that α-galactosyl residues with specific linkages to type II chains are much abundant in the mucous cells and cells related to them, but are lacking elsewhere. Hence, BSA1-B_4 may be useful in determining the origins of a gastric tumour. The pre-treatment with β-elimination had no observable effect on the staining patterns of this lectin, suggesting that the ligand was part of an N-glycan.

It was observed that the staining intensity in chronic gastritic, metaplastic and dysplastic gastric tissues decreased in comparison with their normal counterparts. This reduction of both staining intensity and the proportion of 'positive' cells was dramatically evident in all types of gastric carcinomata. Most specimens examined showed very low percentages of BSA1-B4 positive tumour cells. There were fewer than 5% of all cells in the most densely stained areas and some specimens lost staining completely.

From these observations, It can postulate that the diminution in α-galactosyl glycans containing Gal α1,3 Gal α1,4 GlcNAc- sequences is an early indicator of gastric carcinogenesis, if not an indicator of tumour progression. The biosynthesis of this glycan might be impaired or abolished either by genetic changes, down-regulation of an enzyme (or enzymes), by the physical failure of reactants to come together (e.g. because of a defect in membrane flow) or by a cessation of synthesis of a 'core' protein.

5.2.2.2 VVA

VVA gave a similar staining pattern to that of BSA1-B_4, although the intensity of stain was less. The surface epithelial and the pit cells were stained, but other components did

not bind this lectin. It was noted that the staining intensity of gastritic, metaplastic and dysplastic tissues was slightly increased as compared with normal mucous cells. Neuraminidase digestion decreased the staining intensity of surface and pit cells, both in normal tissues and in precancerous lesions, but the treatment with β-elimination had no effect on the staining patterns and intensity of stain in these tissues.

VVA also showed a very distinctive staining pattern in gastric carcinoma tissues. All types of gastric carcinoma were rarely stained and showed a very low percentages of scattered positive tumour cells. Neither neuraminidase digestion nor β-elimination achieved observable change in the staining intensity or the proportion of positive cells. In other words, during the occurrence and progression of gastric carcinoma, the tumour cells became more anaplastic, the synthesis of glycans containing GalNAc α1,3 Gal β1-termini was severely diminished, probably as a result of the inhibition, inactivation, or cessation of a synthesis transferase responsible for the process. Hence, like BSA1-B_4, may be a possible early indicator of gastric carcinogenesis.

5.2.2.3 SBA

An intense staining in the surface epithelium and the pit cells was observed in most normal gastric mucosa. The gland cells were very lightly stained individually, or in small clusters, or not at all. No stromal staining was seen in any case. Hence, SBA appears to be a good indicator of cells from epidermal origin, but not from mesenchyme.

Neuraminidase digestion diminished the staining intensity of the normal mucosal cells but β-elimination appeared to have little effect, which means that the terminal GalNAc α1,3

Gal β1- or Gal α1-sequences recognised by SBA are probably on N-glycan and have some, possibly indirect, association with sialic acid.

In the three precancerous conditions, the intensity and pattern of staining resemble that of their normal counterparts, and their susceptibility to neuraminidase digestion and β-elimination was as in the normal controls.

The gastric carcinoma cells showed a different staining profile. The percentages of positive cells were strikingly reduced from the well-differentiated carcinomata (65%) through the moderately-differentiated (23%) to the poorly-differentiated (11%). Diffuse gastric carcinomata also showed very low proportions of 'positive' tumour cells. Pre-treatment with neuraminidase and β-elimination dramatically reduced the numbers of positive tumour cells in well-differentiated carcinomata, and had smaller effects in the other types.

The diminishing proportion of SBA-positive tumour cells with increasing anaplasia implies that there is a correlation between the differentiation or maturation of tumour cells and their expression of terminal GalNAc α1,4- or Gal α1- sequences in the abnormal gastric mucins. It also implies that SBA is a 'candidate' indicator for determining tumour progression and differentiation.

A few articles have described the staining patterns of SBA on various gastric mucosal cells (Bur and Franklin, 1985; Macartney, 1986) and controversial results have been reported. In Bur *et al*'s observations, SBA did not normally stain the surface cells, but, after neuraminidase digestion, the cells showed positive staining. However, in

Macartney's report (1986), SBA stained the epithelial cells strongly and stained the gland cells only weakly. This result is in accordance with the findings above.

5.2.2.4 DBA

DBA showed strong selective staining mainly on the surface and pit epithelial cells. It also showed focal staining on gland cells individually or in small clusters. β-elimination did not alter the staining patterns and staining intensity. The results indicate the abundance of terminal N-acetylgalactosamine with adjacent α-L-fucose branches in normal gastric mucosal cells, probably mostly as a part of N-glycans.

In the gastritic, metaplastic and dysplastic tissues, the staining intensity was decreased in most cases relative to normal. It was also affected by β-elimination.

The most important alterations were observed in the carcinoma cells. DBA, as stated above, showed strong ability to combine with the sugar residues of normal mucosal cells but failed to stain tumour cells, implying that the sugar residue GalNAc α1,3 (Fuc α1,2) Gal β1,3/4 GlcNAc β1- was much more abundant in normal mucosal cells, while majority of the tumour cells were not stained with DBA, leading to an extremely low population of positively stained tumour cells. Some variation was seen among cases.

This phenomenon is of interest, because there is such a high contrast in staining pattern and intensity between the normal gastric mucosal cells, which were strongly positive, and the tumour cells, which were rarely and weakly stained. There is a clear change in biosynthesis and expression of DBA ligand during tumourigenesis and/or progression,

which results from a severe decrease of glycosyl transfer. Whether this is of genetic or biochemical origin is unknown.

DBA, a lectin from *Dolichos biflorus*, was purified and characterised in 1970 (Etzler and Kabat, 1970). It specifically recognises GalNAc α1,3 (Fuc α1,2) Gal β1,3/4 GlcNAc β1-sequence and has blood group A specificity (Etzler and Kabat, 1970; Baker *et al*, 1983). In a previous study of gastric mucosa, it has been reported as showing a strong affinity, specifically, for the supranuclear region of the surface mucous cells and the perinuclear region of the parietal cells (Ito *et al*, 1985). This finding is consistent with the observation in this study, in that the strongly stained part of each epithelial cell was the cytoplasm and the supranuclear region in particular. The staining in gland cells appeared more dense around the nuclei of parietal cells. This indicates that the sugar sequence it specifies is usually expressed at a relatively high level. Other investigations provided support to the observations above (Pescheke *et al*, 1983).

The localisation in the superficial epithelial cells of the ligand recognised by this lectin has been proven in animals by electronic microscopy, which showed that this region was in fact the Golgi apparatus (Bergeron *et al*, 1982). This is understandable, in that Golgi apparatus is the main site of glycosyl transfer, of residues such as D-galactose, N-acetyl-D-galactosamine and sialic acid, to the maturing oligosaccharide chains. Other electron microscopic studies have shown a transitional change of staining intensity from the *cis* to the *trans* face reflecting the spatial organisation and segregation of glycans and transferases in the Golgi apparatus (Roth, 1984; Sato and Spicer, 1982b; Pavelka and Ellinger, 1991). Generally, DBA is assumed to bind to the N-acetyl-D-galactosamine residues of the glycoproteins. It was postulated that one or two GalNAc-like units might

exist in the *cis* side of the Golgi cisternae in the surface mucous cells of gastric mucosa (Ihida *et al*, 1991).

The staining on gastric carcinoma tissues by DBA, on the contrary, was shown to be significantly lower than in normal tissues. In Narita's report (1992), the cases of positively stained tissues of either intestinal-type or diffuse-type gastric carcinoma were around 25%, in contrast, normal tissues showed 90% percent stainability. This study, to a degree, was in accordance with the results reported here. It was found that in most cases gastric carcinoma tissues showed very weak or no staining by DBA, only a few tumour cells were stained and these were sparsely distributed in the tissues.

It was also found that the staining by DBA of gastric mucosal cells depended on the blood group specificity of the carrier (Pongchairers *et al*, 1987, Narita and Numao, 1992). In the mucosae from group A or AB carriers, the percentage of 'positive' cases was significantly higher than that from group B or O (H), but blood group specificity did not make much difference in gastric carcinomata. This may be because the blood group substance of A2 has the same glycosyl residues recognised by DBA,

Therefore, DBA is a potentially important marker that could be used to distinguish normal mucosal cells and abnormal cells, both in benign and in malignant lesions of the stomach.

5.2.2.5 CTA

Unlike the other lectins above, CTA only showed staining on surface mucosal cells. The rest of the mucosal components did not stain. In gastritis, metaplasia and dysplasia, the

staining intensity was much reduced and was very weak. Gastric carcinoma cells also tended to show weak positive staining in a small number of tumour foci. High-grade gastric carcinomata had fewer CTA-positive tumour cells (about 5%) than did low-grade carcinomata (over 20%).

The results imply that certain subsets of branched glycans bearing Gal β1,4 GlcNAc β-termini are probably confined to a particular type of mucosal cell (the surface epithelium), hence the glycosyl transferases directing the synthesis of these glycans are also selectively expressed. During tumourigenesis, the functional activity of the transferases may be perturbed at an early stage, but gradually recover with the tumour progression.

5.2.3 Gland cells

Some saccharide ligands were preferentially expressed in gland cells, with less or none being found in the surface and/ or pit mucosal epithelial cells. These were provisionally interpreted to be as follows: **PSA** (α-D-mannose in bi- or tri- antennary, non-bisected complex N-linked sequences); **LCA** (α-D-mannose, similar to PSA); **GNA** (non-reducing terminal α-D-mannose especially mannosyl α1,3 mannose sequences); **NPA** (α-D-mannose especially in mannosyl α1,6 mannose linkage); **UEA-1 or LTA** [α-L-fucosyl terminals, especially L-Fuc α1,2 Gal β1,4 GlcNAc β1- (UEA-1) or clustered (LTA) in the core sequences in N-linked glycans]; **SNA** (NeuNAc α2,6 Gal/GalNAc); **DSA** [Gal β1,4 GlcNAc and (-4 GlcNAc β1)$_2$]; **HPA** (GalNAc); **BPA** (β-D-gal α1,3-GalNAC) and **BSA-II** (D-GlcNAc).

5.2.3.1 PSA

As shown in the results, PSA did not show staining of the surface and pit epithelial cells, but gave strong staining of gland cells. It also intensely stained the stromal components including smooth muscles. This may be because of the cell-specific transferase in gland cells and stromal cells which only or mainly catalyses the mannose addition in non-bisected bi/tri antennary complex N-linked to the glycans. Alternatively, it may be a reflection of the proportion of N-glycan that is non-bisected in various cells and the N-acetylgalactosyl transferase I to III may contribute to the synthesis of the glycans.

A decrease of staining intensity in gland cells was observed in gastritic, metaplastic and dysplastic tissues. This reduction became much more evident in the gastric carcinoma tissues. In well-differentiated carcinomata, 'positive' tumour cells accounted for nearly 76% of the tumour cells. With progression and anaplasia, the number of positively stained tumour cells sharply diminished as about 25% in moderately-differentiated carcinomata, and to 11% in poorly-differentiated and diffuse carcinomata. Signet ring cell carcinomata, however, showed a very high percentage of positive cells (89%).

The results imply that PSA staining possibly detects an early change in N-glycan assembly during gastric carcinogenesis. It may be due to the inhibition of mannosyltransferase activity, or the synthesis of the enzyme itself is suppressed. The high ratio of positive tumour cells in signet ring cell carcinomata may reflect the biosynthesis of a new glycoconjugates.

5.2.3.2 LCA

LCA and PSA have very similar specificity for sugar binding, and as expected, LCA produced a nearly identical staining patterns to PSA in all types of gastric tissues although some differences were noted.

LCA did not show staining of the surface epithelial cells. It stained part of the pit mucosal cells as well as most of the gland cells. In glands, LCA stained chief cells slightly more strongly than parietal cells. Stromal tissues were stained. The specific mannose-containing glycans recognised by LCA were, therefore, localised mainly in gland cells, especially in chief cells, while some of the neck cells also contained a small amount of such glycans.

Decreased staining, relative to normal, was seen in the gastritic, metaplastic and dysplastic tissues. The staining patterns in all the gastric carcinomata were similar to those given by PSA, and showed that well-differentiated carcinomata had a higher proportion of positive tumour cells than other types. With increasing anaplasia, the proportions of positive cells dropped greatly. Signet ring cell carcinomata contained relatively higher proportions of positive cells. The mechanisms responsible for the changes in LCA-binding glycans may well the same as, or very much like, those causing changes in PSA-binding ligand.

5.2.3.3 GNA

A distinctive staining pattern was produced by GNA in normal gastric mucosa: surface epithelial cells were left unstained, neck zone cells were partially stained and gland cells were strongly stained, and stromal tissues remained unstained. It also showed the staining of parietal cells was stronger than that of chief cells.

Hence, the non-reducing terminal mannose, especially that in α1,3 linkage to sub-terminal mannosyl residues, is cell-specific in expression and predominantly occurs in gland cells and, to a slightly lesser extent, in neck cells. The mature surface epithelium and other mucosal components appears to lack glycans containing such non-reducing terminal mannose.

In precancerous lesions, a diminution in staining intensity relative to normal, was observed in most cases. Gastric carcinomata showed a gradual decrease in their percentages of 'positive' cells from well-differentiated carcinomata (31%) through moderately (29%) and poorly (15%) differentiated types. In the diffuse (10%) and signet ring cell types (5%), few cells were stained. This gradation of staining could imply that this change in high mannose glycans is an early event of gastric carcinogenesis and, with tumour progression, further diminution occurs in terminal α1,3 mannose.

5.2.3.4 NPA

NPA showed a strong preferential staining of gland cells, while parietal cells stained more stronger than chief cells. The rest of mucosal cells and stromal components did not bind to this lectin. This staining pattern made the glands in the mucosa very prominent. Hence, non-reducing terminal mannosyl residues, with α1,6 linkage to sub-terminal mannose, were specifically localised to the glycans of gland cells but not of other mucosal constituents.

In metaplastic and dysplastic lesions, the staining intensity was less than in normal mucosa, again, suggesting that changes in high mannose glycans occur in the early stage

of gastric carcinogenesis. This is similar to the changes of PSA-, LCA- and GNA-binding ligands. Gastric carcinomata showed low proportions of 'positive' tumour cells and especially low proportions of stained cells were observed among low-grade carcinomata. This indicates that the synthesis or exposure of α1,6 linked mannosyl residues is altered in gastric carcinogenesis and is further diminished with tumour progression.

From these analyses, it is evident that most glycans bearing terminal α-mannose are expressed in cell restricted manner (i.e. mostly in gland cells and, to some degree, in neck cells). They are altered in those early gastric abnormalities that are regarded as 'pre-malignant' so that the changes in these glycans happen very early in gastric carcinogenesis. They become more apparent with tumour progression and increasing anaplasia, since percentages of tumour cells positive with GNA and NPA were much lower in low-grade tumours. These lectins may, therefore, be useful in determining the origin and the degree of differentiation of gastric carcinoma cells, especially those staining only type of gastric mucosal cells, such as PSA and NPA.

5.2.3.5 UEA-1

UEA-1 and LTA are lectins specific for terminal α-L-fucosyl residues, but with different requirements for the sugar sequences that bear them. UEA-1 strongly stained gland cells, which were mainly parietal cells; chief cells appeared cytoplasmically negative. A few surface epithelial cells also showed weak positive staining with UEA-1. Pit cells were not stained by this lectin. Hence, sequences bearing terminal fucosyl and especially Fuc α1,2 Gal β1,4 GlcNAc β1- sequences, were mainly located in gland cells and in a few surface mucosal cells. The lack of effects of neuraminidase digestion and β-elimination on UEA-

1 staining implies that most UEA-1 ligand was probably upon non-sialylated N-glycans.

Pre-neoplastic lesions showed an increase in staining intensity above normal and the goblet cells of metaplastic tissues had clear cytoplasmic stain. Neuraminidase digestion decreased the staining intensity of all three types of precancerous lesions, implying a structural change in the glycans such that some were partially sialylated or were physically hindered by sialyl residues on nearby glycans. It is debatable whether these changes should be regarded as reactive to inflammation or pre-neoplastic in character.

In the intestinal type of gastric carcinoma, the percentages of positive tumour cells were declined from 93% in well-differentiated carcinomata to 87% and 46% in moderately- and poorly-differentiated carcinomata, respectively. In contrast, most carcinomata of the diffuse type showed very low population of positive cells (less than 5.4%) and signet ring cell tumours did not show any recognisable staining. Neuraminidase digestion showed no effect on the number or intensity of stained tumour cells, but β-elimination significantly decreased the 'positive' staining fractions in all types of gastric tumours and the simple diffuse gastric carcinomata became negative after this treatment.

These results could indicate several types of change in fucosylated glycans which may differ between early alterations in pre-neoplastic lesions, in which sialic acid is involved and later changes in which fucose is lost. There may be a severe fall of fucosyl transferase activity in intestinal type of gastric carcinomata and nearly complete suppress in diffuse type. There may be alternative mechanism responsible for the staining in diffuse-type gastric carcinomata by which the gene(s) directing the synthesis of fucosyltransferase is suppressed or the enzyme is inactivated. The difference in staining

characteristics between the two types of gastric carcinomata may provide an indicator of histogenesis in the subtyping of gastric tumours.

A transition in the type of glycans present in the tumour cells is also indicated in that there is a shift from N-glycan to O-glycan, as showed by the changed susceptibility to β-elimination. This may imply changes in the types of mucin core protein expressed.

5.2.3.6 LTA

LTA differs from UEA-1, in that it binds to fucosyl residues in clusters or in α1,6 linkage to the core sequences of N-glycans. This lectin showed very strong staining of gland cells and left all other mucosal cells unstained. This means that the fucosylated LTA-ligands are markers of gland cells. The β-elimination pre-treatment decreased the staining intensity of the gland cell, showing that at least a proportion of the LTA-binding fucosyl terminals were present in O-linked glycans.

Observations on the staining of precancerous lesions showed that the staining intensity of the gland cells was slightly elevated in gastritis, but remained unchanged in other two lesions. It also stained the metaplastic goblet cells. β-elimination reduced the staining intensity in these lesions and in the goblet cells. It, therefore, is evident that inflammatory factors may have effects on the glycans of gland cells, independently of neoplastic progression.

In gastric carcinomata, the general staining was similar to that seen with UEA-1. The proportion of positively stained cells decreased with increasing anaplasia and very low

proportions were seen in the diffuse carcinomata, while signet ring cell carcinomata showed no staining at all. β-elimination had marked effect on the positivity of staining, leading to severe diminution of staining in all previously stained specimens and diffuse carcinomata was completely lost of staining. An unusual phenomenon was the staining of signet ring cell tumours after β-elimination. They showed over 61% of positive cells in comparison with their previous total lack of stain. This implies either that an O-glycan which had hindered access to an LTA-ligand had been removed, or that the conditions of β-elimination were inducing a conformational change in a fucosylated glycoconjugates.

An explanation for the decreased staining in gastric carcinomata is possibly the downregulation of a fucosyl-transferase, which leads to the reduction of glycans with fucosyl terminals. This may also involve a decrease in N-linked glycans containing such fucosyl terminal sequences and the increase of O-linked glycans, relatively or absolutely.

5.2.3.7 SNA

SNA is a lectin specific for N-acetyl neuraminic acid in α2,6 linkage to galactose or N-acetyl galactosamine. It also binds weakly to β-galactosyl termini. From the results, it appeared that all gastric mucosal cells carried such NeuNAc α2,6 linked sequences and that the only difference among them were quantitative. Gland cells contained much more α2,6 linked sialic acid than the other two types of epithelial cell. The stroma was also intensely stained by SNA.

Neuraminidase digestion greatly diminished the staining of mucosal cells, confirming that the gastric glycans contained terminal sialic acid. β-elimination had no effect on the

staining intensity of mucosal cells, suggesting that the glycans carrying α2,6 sialyl termini were mainly N-linked to the core proteins.

All three types of precancerous lesions showed a decreased staining intensity in comparison with their normal counterparts and their reactions to neuraminidase digestion and β-elimination were similar to those of normal mucosal cells. Tumour cells showed between 55% and 86% of positively stained cells in all types of gastric carcinomata. There was little difference between the intestinal and diffuse types. Neuraminidase digestion led to a reduced proportion of 'positive' tumour cells in all carcinomata. However, β-elimination slightly increased the positive percentages of tumour cells. This may have been because of the removal of O-linked glycans sterically blocking the SNA-binding sequences.

5.2.3.8 DSA

DSA binds to the same types of glycan as LEA and the difference between them is that each lectin has a characteristic rank order of affinity towards them. DSA sequesters Gal β1,4 GlcNAc β1- sequence (especially as clustered branches) rather than (-4 GlcNAc β1-)$_2$, while LEA binds to (-4 GlcNAc β1-)$_2$ rather than Gal β1,4 GlcNAc β1-sequences. In principle, they should show similar staining patterns, but the intensity may vary. they produced such staining patterns here, in that the staining of gastric mucosal cells, while broadly similar, appeared slightly different with the two lectins. LEA gave an overall staining in most mucosal cells, but DSA showed focal staining in all layers of mucosa: part of the surface and pit epithelial cells were stained and most of the gland cells was intensely stained. Unlike LEA, DSA also stained stroma intensely. The

comparison between the staining patterns of these two lectins illustrates the preferential distribution of particular types of glycans. Even two lectins which share the same sugar binding specificity may produce different staining patterns and intensity because of their individual selectivity towards larger glycans.

Neuraminidase digestion increased the overall staining intensity of DSA while β-elimination did not affect the staining. This is probably because of the removal of terminal sialyl residues of branched N-glycans so exposing terminal Gal β1,4 GalNAc β1- clusters.

In the three precancerous lesions, the general staining intensity was decreased slightly, compared with that of normal counterparts. Neuraminidase pre-treatment led to the enhanced staining, but β-elimination seemed to have no effect on this.

High proportions of tumour cells were stained in all types of gastric carcinomata. This fraction was further enhanced by pre-treatment with neuraminidase, so that all tumour cells were stained cytoplasmically or on their membrane or both. β-elimination reduced the staining of the intestinal carcinoma but increased that of the diffuse type.

Although it is difficult to explain the mechanisms and the implications of the above results without further information, they indicate an alteration of the synthesis of those glycans which are the DSA-ligands. This occurs either because they are under-sialylated in the terminus or because their absolute level is changed. The results from β-elimination suggests that, in the intestinal type of carcinoma, there may be an increase of O-linked

glycans carrying DSA-specific sugar sequences. Another mechanism must be responsible for the staining in the diffuse type of gastric carcinoma, since pretreatment with β-elimination increased staining by DSA. The probable explanation of this affect is that an O-glycan was removed which had been causing steric hindrance of the DSA ligand.

5.2.3.9 HPA

HPA is a lectin which specifically recognises N-acetyl galactosamine in sugar chains, but is of a protein family unrelated to SBA, VVA or WFA. In normal gastric tissues it showed a strong staining of gland cells and scattered staining of surface and pit cells. In gland, the staining of HPA was notably preferential and a potential marker for the parietal cells rather than chief cells, as others reported previously (Suganuma *et al*, 1985). These observations are in accordance with other authors who claimed relatively weaker staining in the surface epithelial cells (Macartney, 1986). However, other investigators also found that this lectin stained all components of the mucosa with strong positivity (Fischer *et al*, 1984). It is not clear why various results were achieved. It may because different working dilutions of HPA were used, which led to non-specific staining.

Neuraminidase digestion did not alter the staining of gland cells but slightly enhanced that of surface and pit cells, implying the some N-acetylgalactosamine residues were substituted by sialic acid (e.g. NeuNAc α2,6 GalNAc α1-). β-elimination had no effect, suggesting that the glycans recognised by HPA are mainly N-linked.

In the precancerous lesions, staining with HPA decreased below normal. The goblet cells were clearly stained cytoplasmically and their staining intensity was not affected by either

enzymatic digestion or β-elimination. This shows that the HPA-binding glycans were not substituted with salic acid, but it is not clear whether this was a reactive change or part of a neoplastic process.

High proportions of 'positive' cells were found in the intestinal type of gastric carcinoma, but low ones in the diffuse type. Neuraminidase digestion did not generally alter these patterns, but it did greatly increase the staining positivity of signet ring cells. Beta-elimination dramatically reduced the fractions of positive cells in all types of carcinomata, except for signet ring cells, which increased markedly. These results strongly suggest that D-GalNAc residues of the HPA-ligand are largely in O-linked glycans in most gastric tumours. The increased staining in signet ring cell carcinomata after neuraminidase digestion is probably caused by the removal of sialic acid from other glycans which could block the access of HPA to N-acetyl galactosamine residues.

5.2.3.10 BPA

The staining patterns produced by BPA characteristically showed strong staining in gland cells, but not in surface and pit cells. Among gland cells, those stained were mainly chief cells. Stroma also stained strongly with this lectin. BPA is specific for β-D-galactose α1,3 linked to N-acetyl galactosamine and it can be postulated that such glycans are abundant in the gland cells (chief cells) rather than elsewhere in the mucosa. Neuraminidase had no effect on the staining with BPA.

None of the precursor lesions showed alterations of staining intensity from normal before or after those pre-treatments with neuraminidase or β-elimination. In gastric carcinomata the ratios of positive cells were between 45% to 70% in all types; there was no change

with tumour progression. This implies that the synthesis of glycans bearing β-D-galactose α1,3 linked to N-acetyl galactosamine sequences are not affected and are probably part of N-glycans, without, at least direct, association with terminal salic acid.

5.2.3.11 BSA-II

A clear staining pattern was produced by BSA-II in that only gland cells (parietal cells) were intensely stained while all other components remained unstained. It suggests that the D-N-acetylglucosamine residues recognised by BSA-II are very selectively expressed and are, in this context, almost cell type specific. The stained cells were mainly parietal cells, which were cytoplasmically positive, while chief cells showed unstained cytoplasm, but showed staining of their cell membranes. Staining patterns were unaffected by β-elimination, which indicates that the glycans were mainly N-linked.

The 'precancerous' conditions showed no changes in staining from normal gland cells. In contrast, the 'positive' cell percentages in the true carcinomata were no more than 20% in any type and the staining was completely lost in signet ring cell tumours. The positive cell ratios were further lowered by β-elimination, suggesting that glycoproteins (possibly mucins) containing abundant O-linked glycans were synthesised during tumourigenesis and progression. The low proportions of tumour cells stained by BSA-II may imply two possibilities. First, the decrease of glycans containing D-GlcNAc could occur in the very early stage of gastric carcinogenesis, so that only small proportion of tumour cells retain this phenotype. Second, the tumour cells could mostly be differentiating towards cells of other than glandular type, so that only a subset of neoplastic cells retain the capacity to produce the BSA-II ligand. If this can be solved, BSA-II could have a value in determining the histogenesis and progression of gastric neoplasms.

5.2.4 Low affinity to all mucosal cells

It was evident that some sugar residues were sparse in normal mucosal cells, as revealed by three lectins: **MAA** (NeuNAc α2,3 Gal β1-); **LFA** (NeuNAc and NeuNGlycosyl α2-) and **PTA** (GalNAc and Gal-). However, their staining was variously increased, or not, relative to normal, on the cells of different types carcinoma. These lectins showed that the content of some sugar ligands was y relatively high or massively increased over normal in some tumour cells.

5.2.4.1 MAA

MAA stained very few normal mucosal cells, but stained parts of metaplastic and dysplastic tissues. It gave generally strong staining with all types of gastric carcinoma cells. This indicates that the switching-on of α2,3 sialyltransferase activity occurred at a very early stage of gastric carcinogenesis. The enzyme activity persisted generally during the progression of the tumours, but in some neoplastic cells it was lost or diminished, leading to focal variability and heterogeneity in staining in some of the tumours.

MAA is a lectin purified from the seeds of *Maackia amurensis*, which was first isolated in 1974 by Kawaguchi *et al*. Its sugar specificity, was further studied recently (Knibbs *et al*, 1991). It has a strong affinity for the NeuNAc a2,3 Gal β1- sequence and a requirement for the α2,3 sialic residues. Its specificity has been confirmed by the inhibition of its binding to low concentration of 2,3-sialyllactose (i.e. NeuNAc a2,3 Gal β1,4 Glc), but not other oligosaccharides. It does not bind to the a2,6 sialyl linkage or other sugar sequences.

This lectin, together with SNA and LFA, is sialic acid-specific, but they have different binding requirements for particular linkages in oligosaccharide chains. SNA specifically recognises a2,6 linked oligosaccharides, which specificity is verified by its inhibition by 2,6-sialyllactose (NeuNAc a2,6 Gal β1,4 Glc) (Shibuya, 1987). It does not bind to the a2,3 linkage under the conditions used for histology, but has a low affinity for N-acetyllactosamine. LFA, a lectin from the yellow slug, has a different sugar specificity and which is inhibited by free NeuNAc. It can also interact with saccharides containing N-glycosyl neuraminic acid.

MAA, in this study, stained most tumour epithelial cells in the better differentiated forms of intestinal-type gastric carcinomata, but fewer of the tumour cells in both poorly-differentiated intestinal-type and diffuse-type gastric carcinomata. It is evident that the content of glycans carrying a2,3 linked sialic acid is enormously increased in most tumour cells of all types of gastric carcinomata, especially if the neoplasm is well or moderately differentiated. This implies that the synthesis of terminal sialic acid containing α2,3 linkage is activated at the beginning of gastric carcinogenesis, and this switching-on is probably associated with the cellular differentiation. With increasing anaplasia, tumour cells gradually lost part of the capacity to synthesise such glycans. The mechanisms for the change of glycans are not known. It may be because of the decrease of sialyl transferases themselves or their activities which are altered by unknown factors.

In the stomach, the mucins produced by the mucosal cells contain very little sialic acid, this is not surprising because sialyl glycosides are acid-labile and would be hydrolysed by the high concentration of warm acid in the stomach (Fouad and Waldon-Edward, 1980).

Hence, the a2,3 linked sialic acid, in the sugar chains that MAA recognises, would have a short half-life and would be almost or completely absent in the mucins produced by gastric mucosal cells.

It seems that the α2,3 linked NeuNAc sequences were increased far above normal in all gastric carcinoma tissues, but to a variable extent depending on the degree of differentiation. Unlike normal gastric mucins, which have a low level of sialic acid, gastric carcinoma cells can produce much more sialyl glycosides with a2,3 linkage. This indicates that, with malignant transformation, the synthesis of glycans is altered, so that the net production of NeuNAc α2,3 Gal β1- termini is upregulated. It is not clear that what caused the upregulation resulting in the increase of sialylated glycans, but it must represent either an increase in synthesis or a decline in degradation, probably the former, since the histological boundaries are too sharp to be explicable as resulting from a local zone of higher pH.

5.2.4.2 LFA

For the same reason of staining with MAA in normal gastric tissues, LFA did not show staining in normal tissues, probably also because the free neuraminic acid was degraded. In the precursor lesions, the staining appeared weakly in part of the affected mucosal cells. The goblet cells were clearly stained in the metaplastic mucosa. This may imply that there is a different mechanism regulating the metabolism and expression of N-acetyl neuraminic acid in the goblet cells from that in other mucosal cells, so that goblet cells can continue to synthesise such glycans under inflammatory circumstance.

Gastric tumours also showed a low percentage of positively stained tumour cells. In

well-differentiated carcinomata, no tumour cells were reactive with LFA, but some cells were stained in the moderately and poorly differentiated as well as diffuse-type gastric carcinoma. Beta-elimination slightly reduced the percentages of positive cells, but the overall staining patterns remained. Such results indicate that the well-differentiated tumour cells resemble the normal mucosal cells. With tumour progression, part of tumour cells undergo genetic or biochemical changes, which subsequently lead to changes in NeuNAc metabolism. The absence of staining in signet ring cell carcinomata is notable. It could imply that there is a major derangement of sialic acid metabolism in this neoplasms, so that they have lost the character of most of other types of gastric carcinoma during progression. Alternatively, it may be that the signet ring cells resemble the normal mucosal cells and never express free NeuNAc at any point during their progression into malignancy.

5.2.4.3 PTA

PTA is specific for terminal α- N-acetylgalactosamine and more weakly for α-galactose. The staining on all types of normal mucosal cells appeared very weak and scattered and the surface epithelium did not show staining.

It was noted that the staining in 'precancerous' lesions was slightly enhanced. In well-differentiated gastric carcinomata, there was a high proportion of positive cells, this was significantly diminished in moderately- and poorly- differentiated carcinomata, as well as in diffuse-type carcinomata. The 'positivity' of all types of tumours fell after β-elimination. The reason for such an elevation of terminal N-acetyl galactosamine and/or galactose in the tumour cells and, especially in the well-differentiated type, are not known, but much of it appears to be present as O-glycan. It may represent a

dysregulation of glycosyl transfer or a core protein synthesis (especially mucin core proteins) during gastric carcinogenesis and progression, or might reflect some abnormality of glycan degradation and turnover.

5.3 GLYCAN ALTERATION IN TUMOUR CELLS

Some of the lectins used in this study showed preferential staining of tumour tissues in comparison to the normal gastric mucosa. They showed either a distinct staining not present in normal tissues or an enhanced staining in tumour tissues or a change in distribution. Other lectins showed strong staining in normal gastric tissues, but show no stain or reduced staining in the gastric tumour tissues. Several lectins showed differential staining on different types of gastric carcinomata. Such lectins can potentially be useful in further studying the tumour behaviours during the gastric carcinogenesis and progression. Characteristic staining patterns of the lectins are summarises in outline in table 5.1.

Table 5.1 Lectin staining in different gastric carcinomata

Staining	Intestinal-type Ca			Diffuse-type Ca	
Intensity	Well	Moderate	Poorly	Mucinous	Signet
Increase	PSA, LCA, **MAA,** PTA, HPA, **BPA,**	UEA1, LTA, **MAA,** PTA, HPA, **BPA**	UEA1,**MAA,** PTA, HPA, **BPA**	I-PHA, PNA, **MAA, BPA**	I-PHA, **MAA, BPA**
Decrease	PNA, **VVA, BSA1-B_4, DBA, BSAII**	PSA, LCA, PNA, **VVA, BSA1-B_4, SBA, DBA, BSAII**	PSA, LCA, NPA, LTA, **BSA1-B_4, VVA,** SBA, **DBA, BSAII**	PSA, LCA, LTA, **BSA1-B_4,** NPA, **VVA,** SBA, **DBA,** HPA, **BSAII**	PSA, LCA, GNA, NPA, LTA, **BSA1-B_4, VVA,** SBA, **DBA,** HPA, **BSAII**

From table 5.1, it is evident that two lectins, **MAA** and **BPA**, showed increased staining intensity in all types of gastric carcinomata, indicating that the expression of N-acetyl neuraminic acid with α2,3 linkage to β-D-galactose and D-galactose α1,3 linked to N-acetyl galactosamine sequences was conserved or enhanced in gastric carcinogenesis and tumour progression. There were four lectins, namely, **BSA1-B_4, SBA, DBA** and **BSAII,** which showed extensive diminution or complete loss of staining in the most gastric tumour tissues. This suggests that processes occurred in the tumourigenesis and progression of these gastric carcinomata lead, directly or indirectly, to the diminution or suppression of specific glycosyl transfers.

MAA and BPA were different from BSA1B_4, VVA, SBA, DBA and BSAII in the way that their staining patterns on tumour cells were more intense when compared with that of normal mucosal cells, while the latter five lectins showed lessened staining in tumour tissues. However, this does not mean that all types of gastric carcinomata or all cases in a certain type of carcinoma showed a consistent reaction to each lectin, variations in staining intensity were actually observed in some tumour tissues.

In further comparison to the expression patterns of glycans seen in normal gastric tissues and gastric carcinomata showed the following changes of staining patterns as detected by lectins.

First, diminished staining in tumour tissues over normal counterparts was shown by lectins BSA1-B_4, SBA and VVA. These lectins preferentially stained normal surface and pit epithelium. They showed staining in rare populations of tumours cells with low proportions of 'positive' cells in most types of gastric carcinomata. This implies that the

majority of tumour cells tend towards lessened differentiation (i.e. increase anaplasia) relative to their normal counterparts and have lost, at least in part, the characteristics of normal mature cells, leading to the alteration of those glycans which are normally expressed in mature epithelium.

Second, elevated staining with some lectins was observed in tumour tissues, as shown by MAA and BPA. BPA displayed a preferential staining to most primitive cells (i.e. cells in the neck zone and nearby). The change of its staining pattern is also supports the proposition that tumour cells lose their differentiation to mature mucosal cells. MAA, which rarely stained normal gastric mucosal epithelium, showed increased staining in high proportions of tumour cells in most cases. It suggests that the sialylation of glycans with α2,3 linked sialic acid is greatly enhanced during gastric carcinogenesis and is retained during tumour progression. Hence, MAA may be a very important indicator in gastric oncogenesis.

Third, some lectins retained their staining intensity in tumour tissues at the same level as in normal. The phenomenon suggests that the syntheses of certain glycans are not affected in the process of tumourigenesis and progression. Those glycans are either massively produced, as shown by ECA, MPA, STA and DSA, or retain a low level of expression(e.g. BSA-II). This also implies that the alteration of glycosylation in these tumours is selectively and that there is not a global alteration of glycans during gastric carcinogenesis and progression.

It was also noted that some lectins showed more complex variations in their staining intensity, increased in some tumours and decreased in others, such as PNA, which

showed diminished staining in high grade tumours and increased staining in low grade tumours. PSA, LCA and LTA also showed similar variations. This suggests that tumour cells lose the capacity for synthesising these glycans at some stages of tumour progression and regain that ability at other stages, or vice versa.

Hence, the lectins showing differential staining in tumour tissues over normal may be very valuable indicators in distinguishing normal and tumour cells, the normal from the tumourous gastric tissues and /or determining the degree and form of tumour differentiation. They are worthy of further exploration in order to assess the potential application in studying gastric carcinomata as in studying well as other mucin-producing carcinomata.

5.4 THE EXPRESSION OF SIALYL LEWIS^A AND MUC1 ON VARIOUS GASTRIC TISSUES

Sialyl Lewis^a was rarely expressed in normal gastric epithelium of any type, implying that this antigen was, at least, not major components of gastric glycans. However, its expression increased in the affected cells of 'precancerous' lesions, this suggests that the synthesis of sialyl Lewis^a becomes more active in those proliferative cells, which is probably because the amount and/or the activities of its synthetic enzymes increase.

The increased expression of sialyl Lewis^a was evident in all types of gastric carcinoma, both in cases and in the proportions of 'positive' cells. These results were supportive of previous observations that this antigen was frequently expressed in gastric tumours, especially in the intestinal carcinomata (Murata *et al*, 1992; Kabayashi *et al*, 1993). Doli *et al* (1994) recently reported that sialyl Lewis^a was a cancer-associated carbohydrate

antigen, based on their findings that this antigen was present in both benign and malignant tumours. They also found that the enzymatic activity for sialyl Lewis^a was elevated in tumours. These results suggests that sialyl Lewis^a is a valuable marker in studying gastric carcinogenesis and progression.

Muc1 is the protein of the first mucin gene that was fully cloned. It is a transmembrane protein which has a large molecular weight with a range of 200-500 kDa (Gum *et al*, 1989). It was rarely used in the study of tumours, especially in gastric carcinogenesis. Little is known about its expression patterns in gastric tumours. It has been found that muc1 was seldom expressed in normal and 'precancerous' lesions (Ho *et al*, 1995), this is consistent with the findings in this study.

In gastric carcinoma, the expression of muc1 showed pleophorism in that, low proportions of tumour cells were stained and the 'positive' cells were scattered in the tumour tissues, even in the same glandular structures, not all carcinoma cells were stained. It was noted that the poorly differentiated and diffuse types of carcinoma showed higher proportions of 'positive' cells than those in other types. Signet ring cell carcinomata showed scarcely stained cells. This implies that the expression of muc1 protein is probably an early event, which starts its expression in the well differentiated carcinomata and its expression increases with tumour progression, as shown in low grade carcinomata. The low expression of muc1 in the signet ring cell carcinomata may suggest two possibilities: one is that the MUC1 gene is suppressed during tumour progression, or alternatively, there is a different regulating mechanism in this special type of carcinoma cells. These results indicate that muc1 can be a useful marker in studying the tumour progression in gastric carcinomata.

5.5 BIOLOGICAL SIGNIFICANCE

It has been found, in this study, that a number of lectins showed preferential binding variably to different types of gastric mucosal cells as well as certain types gastric carcinoma cells. Some of these lectins are first time used in gastric study.

It is reasonable to postulate that those lectins which showed the preferential staining on different types of mucosal cells are potentially markers for normal mucosal cells, as demonstrated in results. Lectins which showed differential staining between the normal gastric mucosa and tumours or among different types of tumours may serve as indicators in determining a certain type of gastric carcinomata and the progression of a carcinoma. Those lectins which showed high affinity to gastric carcinomata may also serve as a potential tool in drug-targeting treatment.

5.6 SUMMARY

1. Twenty-seven lectins were used in this study. The results confirmed the previous studies on gastric tissues with lectins and, more importantly, some new lectins were found potentially to be of great use in further research in gastric carcinomata.

2. There were a number of lectins which specifically stained the mucinous epithelial cells (surface and pit cells), other mucosal components were not stained by them. This group included BSA1-B_4, VVA, SBA and CTA, which could be used as markers for gastric mucin-secreting epithelial cells. This is also implies that some saccharide sequences are mainly expressed in glycans in the mature epithelium.

3. There were several lectins which showed selective staining mainly on gland cells and neck cells and most of them showed preferential staining predominantly on chief cells rather than on parietal cells. This included GNA, NPA, UEA-1, LTA, PTA, HPA, BPA and BSA-II. Chief cells were usually stained cytoplasmically, while parietal cells were negative or showed faint membranous staining. Lectins showing preferential staining among gland cells also included PNA, WFA, MPA and STA. This suggests that certain sugar residues are selectively expressed in primitive epithelial cells and cells at early stages of differentiation.

4. There were a number of lectins which only stained mucosal epithelial cells, but none of the stromal tissues. They could, potentially, serve as mucosal epithelial cell markers to determine whether tumour cells were from epithelial origins. These lectins included GNA, NPA, UEA-1, LTA, PNA, BSA1-B_4, MAA, VVA, WFA, SBA, DBA, CTA and BSA-II.

5. MAA showed a generally enhanced staining in all types of gastric carcinomata in comparison with the normal tissues. It could be of value in further studying gastric carcinogenesis and carcinomata from other mucosal epithelial sources.

6. There were a number of lectins which showed particular staining patterns in gastric tumour cells of different types. For details refer to table 5.1..

7. Several other lectins showed strong staining of normal gastric tissues, but showed very low proportions of positive cells, or weak staining, in all types of gastric carcinomata.

These included BSA1-B_4, VVA, DBA and BSA-II. They may also serve as markers or indicators of differentiation in gastric cancer.

8. The mechanisms responsible for the alterations of glycans were proposed and discussed. The alterations of glycans are at genetic level, that is, the genes directing the synthesis of glycosyl transferases or mucin core proteins are activated, so that certain glycans become abundant or shift from N-glycans to O-glycans, because of increased transferase activities and/or more sites for glycosylation; or those normal genes are suppressed, leading to low levels and activities of transferases or few sites for glycosylation. They may be at biochemical level, some transferases function actively, while others are inhibited, by environmental factors, or the degradation of some glycans are changed. It is also possible that the configurational changes contribute to the alterations in glycans.

9. This study confirmed that sialyl Lewis^a could be a useful indicator in gastric carcinogenesis and tumour progression. It was also found the expression of muc1 protein increased in gastric carcinoma tissues, which suggests that muc1 protein is a potential marker in studying gastric oncogenesis and later progression.

CHAPTER 6 LITERATURE REVIEW ON ONCOGENES AND TUMOUR SUPPRESSOR GENES

6.1 ONCOGENES AND TUMOUR SUPPRESSOR GENES: GENERAL

Molecular genetics has provided a new set of approaches to the exploration of the aetiology and pathogenesis of malignant neoplasms. Many studies have used molecular techniques, such as polymerase chain reaction (PCR), and DNA and RNA *in situ* hybridisation, and found much finer and more subtle changes in ploidy, heterozygosity, micro-satellite instability, gene expression, mutation and transfection of DNA molecules than traditional cytological methods.

One of the important roles of these genetic technologies has been to facilitate the discovery and exploration of genes related to tumourigenesis and development, so-called 'Oncogenes' and 'tumour suppressor genes'.

An oncogene is defined as 'a gene whose abnormal expression or altered gene product directly determines the production of the malignant phenotype' (Marshal, 1986). Oncogenes found in viral genomes are termed as 'viral-oncogenes' or 'v-onc', while their counterparts that exist as normal, constitutive genes in normal animal cells are termed as 'proto-oncogenes' or 'c-onc'. However, the term 'oncogenes' is often wrongly used as a synonym for proto-oncogenes in the literature. Another set of genes, which were once considered as oncogenes, but later were proved to have the potential to inhibit the occurrence and progression of a tumour, are called 'tumour suppressor genes'.

There have been over 40 oncogenes and tumour suppressor genes identified in human genomes and many oncogenes have been isolated from viral genomes. A substantial proportion of these latter are derived from non-human neoplasia.

The first oncogene identified, *src*, was found in Rous sarcoma virus (RSV) genome in early 1970's (Vogt, 1971). It was known that RSV had a potential ability to induce the transformation of chicken fibroblasts in cultures, while its close relative, avian leukosis virus (ALV) had very weak transforming potential. This difference afforded a tool with which to search for the molecular basis of the transforming capacity of RSV and this led directly to the

identification of *src* oncogene.

Among many investigators, Duesberg *et al* (1970) found that the RNA of RSV is about 10 kb, which is1.5 kb longer than that of ALV, raising the suspicion that the extra segment contained vital information required for the induction of transformation in fibroblasts. This was later confirmed by Steven Martin (1970) and Peter Vogt (1971).

In Martin's work, RSV was pre-treated with a chemical mutagen before infection of fibroblasts *in vitro*. The cultures were then incubated at 35°C. All transformed cell clones were isolated and further tested for their ability to induce transformation at both 35°C and 41°C. Some mutant clones would not induce transformation at 41°C, but they recovered this ability when returned to35°C. Thus, temperature sensitive (or 'permissive') mutants of RSV were then established. These mutants was able to grow well at both 35°C and 41°C, which implies that mutation by the chemical mutagen only affected their capacity for transformation, but did not affect their replication. Another important implication of Martin's work was that the extra information in the RSV genome was not only required for the initiation of transformation, but was also necessary for the maintenance of this transformation, since he found that the transformation induced by temperature-sensitive mutants at 35°C could be abolished, with reversion to normal morphology, at 41°C and vice versa.

Vogt (1971) isolated transformation-defective mutants of RSV and, by comparative study, found that these mutants lack about 15% of the normal genome and so contain about 8.5 kb. Their transforming ability was totally lost, but they could replicate. This means that wild-type RSV contains about 1.5 kb nucleotides that are not necessary for its replication, but are vital for its transforming capacity.

Therefore, RSV contained a single gene that was required for the transformation of chicken fibroblasts and was later identified as *src*. Soon afterwards, a number of oncogenes were identified from other viral origins. By using part of these oncogenes as probes, their homologous sequences were identified in the genomes of animals and human beings, which are now known as proto-oncogenes or c-onc. Oncogenes are still being found and they are correlated with various human malignancies.

6.2 ONCOGENES IN GASTRIC TISSUES

About nineteen oncogenes and tumour suppressor genes in thirteen families have been studied in gastric tissues and the majority have been found to have some relation to the genesis of gastric carcinomata. These include: c-erbB1, c-erbB2, c-erbB3, c-Ha-ras, c-Ki-ras, c-N-ras, c-myc, L-myc, c-met, TPR-met, p53, bcl-2, c-fos, c-mos, c-yes-1, HST, INT-2, c-fms and pp60src.

6.2.1 The erbB family in gastric tissues

This family has four members and c-erbB2, especially, has long been the subject of oncogene research. Similar results and conclusions, with only slight discrepancies have been reached by several investigators. So far, there is no literature upon the relation of c-erbB4 to gastric carcinomata.

C-erbB-1 is a comparatively new oncogene in the erbB family (Thompson and Gill, 1985; Prigent and Lemoine, 1992). Its product, a 170 kDa glycoprotein, consisting of three domains with a cyteine-rich extracellular domain, similar to c-erbB-2, has been identified as a receptor of epithelial growth factor (EGF) and transforming growth factor-α (TGF-α). Binding of its ligand leads to the dimerisation of the protein receptor that, as a consequence, increases the catalytic activity of the tyrosine kinase of its intracellular segment. This activates a cascade of kinases, thereby enhancing the signal and eliciting cellular proliferation.

It has been found that the overexpression of the receptor occurs in a wide variety of human tumour types, most commonly in squamous carcinomata in various sites, but less commonly in adenocarcinomata, rarely in gastric adenocarcinoma (Nomura *et al*, 1986). Therefore, more evidence is still needed in order to elucidate its functions in gastric carcinogenesis.

There is a substantial literature on c-erbB-2 and its oncoprotein in various human malignancies, including the gastric carcinomata (Yonemura *et al*, 1991, 1992; Uchino *et al*, 1993; David *et al*, 1994; Lee *et al*, 1994). The c-erbB-2 gene, also called Her2 (based on the homology to Human EGF Receptor) or neu (as identified in mouse neuroblastoma), encodes a transmembrane glycoprotein with molecular weight of 185 kDa (p185).

Studies so far confirm that it is closely related to the epithelial growth factor receptor (EGFr), but its physiological ligand has yet to be identified, since neither EGF or TGF binds directly to p185. There are several growth factors which have been suggested as being potentially the ligands for the c-erbB-2 protein receptor, such as 30 kDa, 45 kDa, 35 kDa and 25 kDa glycoproteins. Each of these can bind to p185 and all show biological activity (Prigent and Lemoine, 1992). However, none of them have been finally confirmed as the physiological effector and the functional relationships between these proteins remain to be clarified.

The p185 protein has been immunohistochemically detected as overexpressed in a wide range of human tumours. The mechanism of p185 overexpression appears to be by way of gene amplification. The role of abnormal expression of c-erbB-2 in human tumours is currently uncertain. The findings of studies on this oncogene and its oncoproduct so far suggest that it is a) a prognostic indicator, b) a possible marker to distinguish intestinal and diffuse types of gastric carcinoma and c) an indicator predictive of lymph node metastasis (Falck and Gullick, 1989; Yonemura *et al*, 1991b; Motojima *et al*, 1994; Lee *et al*, 1994).

The majority of the papers consistently show that this oncogene is more strongly expressed in highly differentiated tumour cells and less in cells of low-grade gastric neoplasms (Falck and Gullick, 1989) and is closely related to the depth of tumour invasion into the stomach wall. It is claimed that the expression of c-erbB-2 determines the prognosis of the patients and can be considered as a prognostic indicator. These studies have employed a diversity of probing techniques, ranging from immunohistochemical staining and Southern immunobloting to PCR, and reveal that an enhanced expression of c-erbB-2 is seen in advanced rather than early gastric carcinomata. In Lee's report (1994), the expression of c-erbB-2 (Her-2/neu) was also found to be associated with the intermediate or high grade of tumour and that the patients with these types of gastric carcinomata had longer survival than average.

Serological analyses also provided evidence of the overexpression of c-erbB-2 with elevated several anti-c-erbB2 antibodies in gastric cancer patients(Yonemura *et al*, 1991b). Other prospective studies found that the survival rates of the gastric carcinoma patients whose tumours express this oncogene was significantly lower and that the death rate was five times higher than those without overexpression. This suggests that c-erbB-2 correlates with tumour stage and the depth of invasion and may be an independent prognostic factor (Motojima *et*

al, 1994).

However, not all findings have supported this conclusion. In their long-term follow-up study, Tateishi *et al* (1992) found that the five-year survival rates of gastric cancer patients with or without the expression of c-erbB-2 were 57% and 59%. They then claimed that the expression of this gene might *not* serve as a prognostic factor. On the other hand, they noted that there are differences of c-erbB-2 expression in different types of gastric carcinomata. Thirty-nine percent of cases of papillary carcinoma were positive, as against 17% of well differentiated, 5% of moderately differentiated and 4% of undifferentiated tumours. They suggested that c-erbB-2 expression could be used for subtyping gastric carcinomata.

Others came to similar conclusions after studying the differential expression of c-erbB2 in the intestinal and diffuse types of stomach carcinoma. Jaehne *et al* (1992) found an elevated expression of c-erbB-2 in well and moderately differentiated gastric carcinomata of intestinal type, which was significantly higher than its expression in the poorly differentiated and diffuse types of gastric carcinomata. In Lee *et al*'s report (1994), among 56 cases of gastric carcinoma, 14 (25%) showed intensely positive nuclear staining for Her-2/neu (c-erbB-2) and all of these were of the intestinal type. Twenty-one (37.5%) showed positive cytoplasmic staining and among them 7 were of the diffuse type.

Overexpression of c-erbB-2 is said to be correlated with lymph node metastasis. In their study, Yoneruma *et al* (1991) claimed that they could not correlate the expression of c-erbB-2 in early gastric carcinomata with other clinical factors, but only with a risk of local and distant lymph node metastasis.

In summary, despite continuing debate, the expression of c-erbB-2 in gastric carcinoma appears to be of great importance in terms both of its prognostic and of its differential diagnostic values, although more information is still needed to clarify its role in the genesis and biology of gastric carcinoma.

C-erbB-3, a newly cloned member of erbB family (Sanidas *et al*, 1993; Katoh *et al*, 1993), and its product, 160 kDa glycoprotein, have attracted far less attention in respect of gastric

tumourigenesis, as compared with c-erbB-2.

This oncogene encodes the peptides of a glycoprotein of 160 kDa, of which the extracellular domain is similar overall to that of EGFr with 60% sequence homology. This is lower than that of p185 which has 82% homology to EGFr of c-erbB-1 (Prigent and Lemoine, 1992; Sanidas *et al*, 1993). It has been proven that the expression of this protein is limited to the tissues of epithelial or neuroectodermal origins (Katoh *et al*, 1993). A functional ligand for c-erbB-3 has not yet been identified and therefore the role of this protein in carcinogenesis remains unclear.

Immunostaining of gastric tissues for c-erbB3 showed that in normal tissues, only the upper third of the mucous epithelium and the parietal cells were strongly positive in restricted areas. In gastric tumours, most of the cells of all types of gastric cancer were positively stained (Katoh *et al*, 1993). There was no significant difference among the histological subsets of tumours or among any other clinical parameters. The only feature observed was that the staining intensity of adjacent tissues, though considerable, is lower than in the tumourous tissues. Therefore, further evidence is required to determine the role of the c-erbB-3.

6.2.2 The ras family

There are three members of ras oncogene family: Ha-ras, Ki-ras and N-ras (Land *et al*, 1983; Barbecid, 1987, O'Hara *et al*, 1986). All encode a group of closely related 21,000 dalton proteins (the p21 proteins), which anchor to the cytoplasmic surface of the cell membrane and bind guanine nucleotides with high affinity. These proteins show a low guanosine-triphosphatase activity (Barbecid, 1987) and so are also as classed as GTPase. They are thought to be the variants of so-called 'G protein', which is part of the signal transduction system, which affects cellular proliferation among other functions. So far, there have been over 50 members identified in the p21 *ras* superfamily. The molecular structures are well understood.

A number of review articles have covered almost every aspect of p21 *ras*, including its tissue distributions, its functions and the mechanisms of its action in the signalling transduction pathways. What follows is a very brief summary of the events of p21 *ras* activation and the resultant effects.

The activation of p21 follows the binding of a ligand to growth factor receptors (for example, the c-erbB-1 oncoprotein), with activation of receptor tyrosine kinases in the intracellular domains of the receptors. This leads to the activation of a guanine nucleotide exchange factor, which results in the conversion of p21 into the active, GTP-bound form. This activated p21 interacts with other protein kinases (such as raf1) and induces the activation of a kinase cascade, triggering a series of cellular responses, such as cellular proliferation, differentiation and inter-cellular reactions.

The amplification of ras genes and the detection of their expressed products, the p21 protein, has been clearly shown in a wide range of cells or tissues and the correlation of overexpression of p21 ras with various human malignancies is well established (Land *et al*, 1983; O'Hara *et al*, 1986; Bos *et al*, 1986; Barbecid, 1987). As a result, efforts have been made to identify mutated *ras* genes in human gastric carcinomata and these have been very productive.

Alterations have been described at codon 12 of Ha-ras and Ki-ras and codons 13 and 61 of N-ras (O'Hara, 1986, Bos *et al*, 1986; Nishida *et al*, 1987). Studies have been carried out on several types of gastric tissue ranging from normal through metaplastic and dysplastic, to frankly carcinomatous tissues, in an attempt to elucidate the role of the *ras* genes (Czerniak *et al*, 1989; Nakajima *et al*, 1989; Carneiro *et al*, 1992; Teh and Lee, 1993). The results of these studies were as follows: *Ras* gene and its encoded product (p21) are rarely expressed in normal gastric tissues, but are usually strongly expressed in the inflamed, metaplastic and dysplastic mucosae as well as in well-differentiated gastric carcinomata. They were also found in tissues adjacent to tumours, especially those with metaplastic and dysplastic lesions. However, a significantly *low* level of immunocytochemical stain for p21 was observed in poorly-differentiated tumours. This implies that *ras* oncogenes are expressed as very early events in gastric carcinogenesis and may play some part in the initiation of gastric malignancy. It has also proposed that the expression of p21 protein could be used as an indicator to discriminate the diffuse type of carcinoma from the well- and poorly-differentiated intestinal types of carcinoma (Teh and Lee, 1993).

Furthermore, there are some suggestions from studies on the mutation of codon 12 of c-Ha-

ras that the expression of p21 can be related to remote metastasis and the survival rate of gastric cancer patients. Immunostaining for anti c-Ha-ras p21 protein provided supportive evidence for this (Yoshida *et al*, 1988). A study on the expression of c-Ki-ras p21 also showed that the appearance of this oncoprotein can distinguish early gastric cancer with low immunohistochemical staining, from its advanced counterpart, with relatively high levels of stain. It was assumed that c-Ki-ras p21 expression can be a prognostic indicator, even though others have drawn the opposite conclusion (Teh and Lee, 1993).

It is reasonable to conclude that the overexpression of p21 is most likely to be an early phenomenon of gastric carcinogenesis, but there remains a lack of certainty as to whether it is the cause or the result of gastric oncogenesis. It may be an indicator of tumour progression and poor survival rate.

6.2.3 The myc oncogenes

There are three members in the myc oncogene family: c-myc, N-myc and l-myc. Since c-myc has been shown to have a relation to gastric carcinogenesis, emphasis is put on this oncogene in what follows and reference will be made to N-myc and L-myc only where appropriate.
C-myc is normally located at the terminus of the chromosome 8 and in malignant neoplasms it can be translocated to the area encoding immunoglobulin heavy chain on chromosome 14 (Prins *et al*, 1993; Packham and Cleveland, 1995). It is now known that c-myc encodes two main types of oncoproducts (Evan *et al*, 1992; Meichel *et al*, 1993), c-myc-1 and c-myc-2, but their structures are still poorly understood. Recently, a third c-myc oncoprotein has been identified and named as delta-c-myc (Zoidl *et al*, 1993).

These proteins are nuclear phosphoproteins which act as transcription factors and can bind, in the form of heterodimers with Max (another oncoprotein), to DNA sequences containing the core hexanucleotide sequence CAC(G/A)TG. They thereby activate transcription of several other genes. Interestingly, when the Max protein itself binds to DNA, there is a resultant downregulation of the same genes as are activated in the combined heterodimer (Kato *et al*, 1992).

It has been also found that the c-myc proteins show 'non-specific' binding to DNA, while N-myc (expressed in neuroblastoma) and L-myc (expressed in lung cancer) lack the domain

which causes this. It has been presumed that this domain may function in regulating the kinetics of specific DNA binding. Further studies have shown that the main form of c-myc oncogene protein, c-myc-1, was decreased or diminished in neoplastic tissues, while another c-myc protein, c-myc-2 was predominant. It is now believed that c-myc-2 confers tumourigenic potential. This suggests that c-myc-1 functions as a 'checking point' for c-myc-2, to prevent certain cells from becoming tumourigenic. The c-myc proteins were expressed at low level in quiescent cells, but at comparatively high level in proliferating cells. Thus, this oncogene may closely relate to cell growth and differentiation and has a role in programmed cell death (apoptosis) (Packham and Cleveland, 1995).

The c-myc oncogene and its oncoproteins were expressed in some cases of gastric carcinoma (Ninomiya *et al*, 1991), but there were no differences between the histological types or clinical stages. The expression of myc proteins is found less frequently than that of their mRNA in the early stage of the carcinomata, which implies that the expression of the oncogene products may be a relatively late event, but the activation of the oncogene occurs earlier and has some undefined role in gastric carcinogenesis. Other studies have further found that this oncogene is related the distinction between non-invasive and malignant lesions. It has also been suggested that it may be a distinguishing feature to discriminate between adenoma and well-differentiated adenocarcinoma, since lesions positively stained for c-myc mRNA were later histologically proven to be gastric malignancy (Tatsuta *et al*, 1994).

6.2.4 Bcl-2

The discovery of Bcl-2 was the result of studies on chromosomal translocation between 18q21 and 14q32. Its name derives from the B-cell lymphoma/leukaemia-2 gene. Bcl-2 is normally located on the segment 18q21.3, in a telomere to centromere orientation, and possesses three exons. Its oncoprotein is of 25 kDa molecular weight. The function of Bcl-2 has been correlated with programmed cell death (PCD) or apoptosis (McDonnel *et al*, 1993; Hawkins and Vaux, 1994; Boise *et al*, 1995; Craig, 1995) and it is considered to act as a critical intracellular regulator which prolongs the cell's life-span by down-regulating apoptosis. Although this blockage of cell 'suicide' is not directly oncogenic, elevated expression of Bcl-2 can increase the risk of secondary changes of other oncogenes or tumour suppressor genes (Hawkins and Vaux, 1994).

The Bcl-2 protein occurs in the nuclear envelope, parts of the endoplasmic reticulum (ER)

and in the outer mitochondrial membrane. Its mitochondrial location has led to a number of studies to examine whether oxidative phosphorylation could be involved in the action of the protein. However, current evidence is against any requirement for oxidative phosphorylation either for the induction of apoptosis or for Bcl-2 to function as a suppressor of cell death. The roles of those forms of Bcl-2 protein which are located in the nuclear membrane and the endoplasmic reticulum have not yet been studied in detail. The action of this protein in the PCD remains to be clarified. (Chen-Levy and Cleary, 1990; Korsmeyer, 1992).

Recently, other Bcl-2-related proteins have been found and the genes encoding them have been termed Bcl-X, Bax and mcl-1 (Reed, 1994). They have varying sequence homology with Bcl-2 protein and are of various molecular weights. It has been suggested that Bcl-X and Bax act in opposition to Bcl-2. Bax, a 21 kDa (192 amino acids) isoform of Bcl-2, can combine with Bcl-2 to form heterodimers, it thereby inactivates the function of Bcl-2. However, further evidence is needed to clarify whether Bcl-2 functions in a homodimeric or oligomeric form for the suppression of apoptosis.

The phenomenon whereby bcl-2 confers a survival advantage upon cells by inhibiting apoptosis has been observed in a diversity of human neoplasms and, in particular, in B-cell lymphomata. There is some evidence for its being related to gastric cancer. In such putatively precancerous lesions as intestinal metaplasia and dysplasia, this gene appeared to be expressed more abundantly in those areas with actively proliferating cells. These findings support the proposition that aberrant expression of bcl-2 may play a role in the development of gastric carcinoma by delaying apoptosis and extending cellular proliferation (Lauwers *et al*, 1994). More direct studies have proved that there is overexpression of bcl-2 in poorly differentiated gastric carcinoma, but not in well-differentiated forms, accompanied by loss of heterozygosity (LOH) in those neoplastic tissues, which show a high degree of differentiation. This means that bcl-2 overexpression has implications for the development of low-grade tumours (Ayhan *et al*, 1994).

6.2.5 P53 tumour suppressor gene and p53 protein

The gene for the phosphoprotein p53 is one of the most widely studied tumour suppressor genes and its product p53 protein has been the subjects of several reviews (Crawford, 1985; Oren, 1985). The activation of p53 gene in human tumours was first documented by

Vogelstein *et al* (Crawford, 1985) and the gene was soon identified.

P53 gene, like the Rb gene, is now well recognised as a tumour suppressor gene and is proven to be recessive (Finlay *et al*, 1988). Its translational product, p53 oncoprotein, is a nuclear phosphoprotein of molecular weight of 53,000 daltons, of fairly well-defined structure and function.

P53 gene was initially considered to be an oncogene, because p53 protein was found to be expressed only at low concentrations in normal cells, but at significantly high levels in SV40-transformed cells. Therefore, it appeared, at first, that elevated p53 protein levels were responsible for cellular transformation and so it was thought to be an oncogene. This proposition was further supported following isolation and molecular cloning of p53: overexpression of these cloned p53 genes could induce cellular transformation in gene transfer assays. However, this simple model was soon confounded.

Further studies documented that the p53 clones that had initially been shown to induce transformation were the *mutant* forms of p53 rather than the wild type p53. The normal p53 gene lacked transforming activity and overexpression of it inhibited cellullar transformation rather than inducing it. This indicated that p53 gene was a tumour suppressor gene rather than an oncogene.

These paradoxical observations led to an alternative interpretation for the transforming activity of p53 mutated proteins. Since normal p53 protein inhibits cellular transformation, but the overexpression of its mutants has the opposite effect, it was suggested that these p53 mutants acted by blocking the action of normal p53. This suggestion was supported by the fact that normal p53 proteins form oligomeric complexes as functional units, while the combination of mutant proteins with normal p53 leads to formation of non-functional hetero-oligomers in which the normal p53 protein is sequestered and so becomes inactivated.

The p53 gene is located at 17q13 and the wild-type (normal) human p53 protein is composed of 393 amino acids and structurally resembles a transcriptional factor. It is present in all normal nuclei at a very low concentration of a few thousand molecules per nucleus and it is essential for cellular growth and division. The three dimensional structure of p53 protein has

been derived and this structure can be correlated with its function. There are five domains within the primary amino acid sequence of p53 proteins, which are highly conserved. The normal half-life span of p53 is very short, being about 10-20 minutes and always less than 30 minutes. Mutant p53 proteins have longer half-lives of up to several hours and so their concentration usually increases reaching about 10,000 molecules per nucleus (Oren *et al*, 1981).

P53 gene has frequently been found to be deleted in carcinomata of colon and rectum. More importantly, the remaining copies of p53 genes almost always contain mutations. These lead to the amino acid substitutions in p53 proteins, hetero-oligomer formation and hence the inactivation of normal p53 proteins. Deletions and mutations of p53 genes occur in a wide range of human tumours, including carcinomata from breast, oesophagus, liver, bladder and ovary.

The normal p53 proteins can inhibit the growth of tumour cells *in vitro* and reduce the efficiency of transformation induced by other oncogenes such as myc and ras. The p53 proteins are nuclear and this is presumably the primary site of their function in suppressing cellular growth. A loss of functional p53 proteins has been provided by the introduction of anti-sense mRNA (Shohat *et al*, 1987) or the microinjection of antibodies directed against p53 proteins (Mercer *et al*, 1984). Both prevent cellular DNA synthesis in response to growth factors, thus providing evidence that supports the role of p53 proteins in suppressing cell growth. It has been experimentally proven that p53 protein can suppress cellular transformation and reduce plating efficiency by forcing cells into G1 arrest, which is considered as the basic physiological mechanism by which the wild-type p53 proteins block cellular growth. Therefore, *either* deletion *or* mutation of the p53 oncogene can lead to the overgrowth of cells and, eventually, to the occurrence of a malignant neoplasm. Studies on the genetics of human tumours produce evidence strongly supportive of this proposition.

In other tumours, p53 is underexpressed because of gene loss and so is more directly involved in oncogenesis. Hence, both elevated histochemical expression of p53 protein and its absence are indicators of neoplastic potential.

There have been several studies on this gene and its products in gastric carcinoma. In general,

the increase of immunocytochemically detectable p53 proteins is an indicator of an elevated level of mutant p53 proteins and hence of diminished normal functional p53. This is a condition for an increased risk of malignancy. Likewise, the total absence of p53 gene and proteins also lead to an increased risk of malignancy.

In stomach, it was found that alterations of the p53 gene occurred in 37.5% cases of metaplasia, in 58.3% of cases of dysplasia and 66.7% of cases of carcinoma, suggesting that p53 gene mutation is one of the earlier events in gastric carcinogenesis (Shiao *et al*, 1994). Evidence from other studies also implies that both p53 gene deletion and mutation are common events in gastric carcinoma with incidence rates ranging from 30-60% (Kim *et al*, 1991; Seruca, 1992; Renaut *et al*, 1993; Soussi *et al*, 1994). Fukunaga *et al* (1994) in their immunohistochemical study, found that the positive staining for p53 proteins was significantly higher in the intestinal type of gastric carcinoma than was that in the diffuse type. They suggested that the p53 gene mutation was not a rare event and might participate in the aetiology of intestinal type carcinoma of the stomach. Other studies have shown that the p53 gene is usually uniformly expressed in all layers of neoplastic cells, whether superficial or more deeply infiltrating, with only a few slight exceptions. This argues that all the tumour cells are derived from the same clone (Cho *et al*, 1994).

The expression of p53 protein has been examined histochemically on various gastric tissues (Fugunaga, 1994). The results showed that this oncoprotein is expressed less frequently in normal and chronic inflammatory gastric mucosae, but more frequently in dysplastic and carcinoma tissues. This implies that the mutation of p53 is possibly an early event in the gastric carcinogenesis, as it appears in excess over normal in precancerous gastric lesions.

6.2.6 The c-fms gene

The c-fms proto-oncogene, has been shown in several studies, to be closely related to the cell cycle (Lee, 1992). Its product, is colony stimulating factor-1 (CSF-1) receptor. It was suggested that both gene and protein down-regulated cell growth by halting it at the G1 phase of the cell cycle.

The c-fms oncoprotein is expressed both in primary tumours and also in the metastases of many human malignancies such as carcinomata of breast, cervix and stomach (Storga *et al*,

1992). It has been suggested that there is a correlation between the expression of c-fms in primary tumours and in their metastases. Its expression appears to be a late event of tumourigenesis.

6.2.7 The c-mos gene

The human c-mos proto-oncogene was cloned in the early 1980's after its viral counterpart v-mos has first isolated from the Moloney sarcoma virus genome (Swan *et al*, 1982; Prakash *et al*, 1982; Papkoff *et al*, 1986). It was predicted to be single coding exon which encoded a serine/threonine kinase. The protein has various molecular weights according to its several origins. Even from human tissues, it appears in two forms: 40 kDa and 37 kDa. The reason for different sizes of the proteins expressed remains unknown.

Transcripts of c-mos have been shown mainly in the reproductive system, including oocytes and ovary, but they also appears in other tissues with very low levels (Yew *et al*, 1993; Pal *et al*, 1994). The gene is rarely expressed in gastric carcinoma tissues, as assesses by using Southern blotting. No conclusion has been drawn as to its function. It appears to be a late event of gastric carcinogenesis and progression, although further evidence is still needed (Ranzani *et al*, 1990).

6.2.8 The c-met oncogene and its product

There have been many articles on this oncogene, its structure and the possible roles of its product, which is the hepatocyte growth factor/scatter factor (HGF) receptor (Bottaro *et al*, 1991; Naldini *et al*, 1991). The c-met gene encodes a transmembrane tyrosine kinase, which can be activated through the binding to its ligand, HGF, a unique cytokine able to stimulate both cellular proliferation and spreading. HGF is synthesised by mesenchymal tissues and it may act as a mediator in stromal-epithelial interactions. The activation of c-met oncoprotein receptor leads to both cell growth and movement.

The expression of HGF receptor has been demonstrated in a range of carcinomata as well as sarcomata. Prat *et al* (1991) found that this receptor was overexpressed in several kinds of carcinomata, including gastric carcinoma, giving up to 50% of positive cases. They observed that the immunocytochemical staining for c-met oncoprotein in normal tissues was generally less intense than in tumours. This implies that c-met plays a role in gastric carcinogenesis by altered expression of the HGF receptor, and might well influence metastatic potential.

6.2.9 C-yes and c-src

The c-yes proto-oncogene encodes a protein tyrosine kinase, p62-yes (c-yes), which belongs to src family of non-receptor-type tyrosine kinases (Brickell, 1992). C-src directs the synthesis of a 60-kDa protein (pp60-src or c-src). Both c-yes and c-src are located on chromosome 11 (Sukegawa *et al*, 1987; Brickwell, 1992). They share extensive sequence homology and are supposed each to have unique domains that mediate the interactions specific to each kinase. The structures of these two genes and their oncoproteins are partially known, but their functions are still unclear.

There is evidence that they may upregulate the cell growth and proliferation, at least in cells of neuritic origin (Pyper and Bolen, 1989; Tsukita at al, 1991) and it has been shown that the phosphorylation of the oncoproteins inactivates them. Hence, it is possible that the mechanism responsible for the abnormal function of these two oncoproteins involves an alteration of phosphorylation (Brickell, 1992).

Altered expression of these oncoproteins have been found in gastric carcinomata (Seki *et al*, 1985; Takekura *et al*, 1990). One study showed that the amplification of c-yes gene was four to five times higher than normal in malignant tissues, but no amplification was found in tissues adjacent to tumours (Seki *et al*, 1985). This suggests that c-yes may be involved in the onset and/or progression of gastric carcinomata. The tyrosine kinase activity of c-src was measured in gastric carcinomata and 50% of the samples showed increased kinase activity (Takekura *et al*, 1990). However, the author found that the expression of c-src was not consistent with the measured kinase activity and so suggested that the elevated kinase activity probably originated from a post-translational change. The role of the gene in gastric carcinogenesis remains unclear.

6.2.10 The Rb tumour suppressor gene and its product

The retinoblastoma (Rb) gene is one of the best characterised tumour suppressor genes. It is located on the chromosome 13q14, was the first tumour suppressor gene identified, and serves as the prototype for this class of genes. Its oncoprotein, Rb protein, has a known structure of 928 amino acids and a molecular weight of 110 kDa (Pickley and Lane, 1994; Hooper, 1994). The p110 Rb protein plays an important role in cellular proliferation, and differentiation as well as in carcinogenesis.

This protein can exist in the nucleus in both hyper- and hypo- phosphorylated forms, the ratio of which varies at different stages of the cell cycle. The hypophosphorylated Rb protein appears through most of G1, while the hyperphosphorylated form is found in the late G1, S, G2 and M phases (Riley *et al*, 1994). This suggests that Rb protein functions in regulating the cell cycle through a phosphorylation mechanism which switches at the beginning of and near the end of the G1 phase.

Experiments have shown that the hypophosphorylated Rb oncoprotein can inhibit progression of the cell cycle and lead to arrest in G1 phase (Riley at al, 1994; Yen and Varvayanis, 1994; Hatakeyama *et al*, 1994). The different forms of Rb protein correspond to its state of activity: the hypophosphorylated Rb protein is an activated form, which is responsible for its normal function, while phosphorylation causes functional inactivation of Rb protein (Knudsen and Wang, 1996). This phosphorylation process is now believed to relate to the cyclins (cyclin A and E, for example) which regulate the specificity and activity of cyclin-dependent kinases (cdk). Evidence has been obtained, *in vitro*, that the cyclins A and E are critical for the hyperphosphorylation of the Rb protein in late G1 phase (Lin and Wang, 1992; Ewen , 1994; Hatakeyama at al, 1994; Knudsen and Wang, 1996).

The function of Rb protein is achieved through its binding to nuclei by way of its combination with a nuclear partner protein or proteins, (termed as the 'nuclear anchor'). Thus, any factors which affect this binding or the structure of Rb protein, and especially the crucial binding sites, can lead to a failure of the protein to arrest the cell cycle, so causing unregulated cellular overgrowth and a risk of malignancy. It has been found that some viral proteins, such as adenovirus E1A, can bind to Rb protein, but not to its mutant (Howe and Bayley, 1992). It may be inferred that either the competitive sequestration of Rb protein by proteins other than its nuclear anchor or chromosomal mutation can cause the loss of the biological function of Rb protein.

Rb protein is detectably expressed in most types of cells that are proliferating or postmitotic (i.e. cells in the cell cycle), while quiescent cells, which have left the cycle, seldom show a detectable content of this protein.

Abnormal expression of Rb occurs in a wide range of human tumours (Higashiyama *et al*, 1994; Cox *et al*, 1994; Wright *et al*, 1995). However, there were few reports of this oncogene and its protein in relation to the gastric tissues, which will be discussed in chapter 9.

6.2.11 INT-2 and HST

These two oncogenes possess similar characteristics in that their oncoproteins both belong to the fibroblast growth factor family, which contains at least nine members (Dickson *et al*, 1989).

The INT-1 and INT-2 genes were first detected in 1983, when Peters *et al* were analysing viral carcinogenesis in mouse mammary glands. They found that, in some tumours, a mouse mammary proviral DNA had integrated and this was always into the same chromosomal region of 24 kb (Peters *et al*, 1984a, 1984b). This region was then named and was thought to be a putative oncogene, which was virally deregulated and led to abnormal cellular growth. Its human homologue was soon analysed and is now known to consist of three exons on the band q13 of chromosome 11 (van Ooyen *et al*, 1985; Casey *et al*, 1986; Law *et al*, 1987; Dickson *et al*, 1989). Its oncoprotein contains 239 amino acids with a molecular weight of 27 kDa.

INT-2 has not been found to be expressed in adult tissues, but occurs in embryos and particularly in their primitive mesodermal tissues from day 7 to 10 during embryogenesis of the mouse (Jakobovits *et al*, 1986). It was then suggested that this gene product served as a growth factor in the early development of embryos by stimulating mesodermal cell migration and tissue induction. In humans, this gene is expressed in some teratocarcinoma cell lines and in a proportion of breast carcinomata, thereby reflecting the immature character of many malignant tissues (Sugimura *et al*, 1990; Theillet *et al*, 1989).

In 1986 by Sakamoto *et al* reported HST in the analysis of DNAs derived from human stomach carcinomata, using the NIH 3T3 focus assay (Sakamoto *et al*, 1986). In 21 samples analysed, 3 were shown to carry a transforming activity upon transfection into NIH 3T3 cells. The transforming sequence was then called HST for Human STomach cancer. It has been found that this gene appears in many kinds of human malignancies.

It is also located on human chromosome 11q13 and separated from INT-2 by a sequence of 40kb (Sugimura *et al*, 1990; Yoshida *et al*, 1991; Hagemeijer *et al*, 1991). These two genes seem always to be co-amplified. HST encodes for a protein of 206 amino acids with 40% sequence homology to the INT-2 product (Yoshida *et al*, 1991). The function of this protein is not yet well established, although some have considered that it might play an important role in the early development of embryos and possibly be involved in human carcinogenesis.

As stated above, the co-amplification of INT-2 and HST oncogenes is a very common event and occurs in various human cancers such as oesophageal, breast, bladder, liver and gastric carcinomata. In stomach, the co-expression rate is reported to be relatively low (Tsuda T *et al*, 1989). These genes possibly participate in carcinogenesis by multi-alterations of oncogenes, although further investigation is necessary to clarify their functions.

In general, the oncogenes listed above and their oncoproteins have individual characteristics and functions in relation to the cell cycle, proliferation and differentiation. However, this does not mean that each functions entirely without involvement of other gene or proteins. In fact, they influence or interact with each other in a complex network of cascades. Any imbalance in their ratio or change of functions may lead either to promotion or suppression of cell growth. What is important is that control of cell proliferation can be lost and that could easily lead to tumourigenesis.

The discovery of oncogenes provides a powerful approach to the analysis of the mechanisms whereby cancer occurs and develops. The studies so far have been very fruitful even though there is still long way to go in understanding the processes by which any cancer arises.

6.3 PCNA AND CELL CYCLE

The concept of the cell cycle was first proposed by Howard and Pelc in 1951 (Baserga, 1981). It was defined as the period between the midpoint of mitosis and the midpoint of the subsequent mitosis. In each cycle, replication of DNA occurs during the synthetic or S phase, which is preceded by a period, the first gap, G1 phase. After DNA synthesis, the cell enters another period of apparent inactivity, designated the second gap phase or G2. Then the cell proceeds towards the mitotic or M phase.

Living cells can be divided into three categories (Leblond, 1964). One group contains actively proliferating cells which are passing through the various phases of the cycle and leads to the cell reproduction. The second population consists of cells which have left the cycle, have differentiated and are destined to die without further division. The third population comprises cells which have temporarily left the cycle and remain in a resting state (designed as G0 phase), but ready to re-enter the dividing population in response to a suitable environmental stimulus.

PCNA is the 36-kDa non-histone nuclear protein identified as the auxiliary protein of DNA polymerase-delta, which is required by the DNA polymerase-delta for the catalytic activity of the enzyme (Bravo, 1987). Its expression is associated with the S phase (DNA synthesis) of the cell cycle (Celis and Celis, 1985), with maximal concentrations detected in the late G1 and S phases (Celis *et al*, 1984). In other word, the appearance or elevated expression of PCNA implies that the cell is in the cell cycle and undergoes an active DNA synthesis, thereby, it is a ideal marker to monitor the cellular state of the cell.

The aims of this study are in attempt to disclose the expression patterns of Rb and other onco- and tumour suppressor genes and compare with the patterns of lectin staining, thereby to explore the roles of these genes in the gastric carcinogenesis.

CHAPTER 7 MATERIALS AND METHODS

7.1 MATERIALS

7.1.1 Gastric tissues

All specimens were selected and drawn from the archives of Pathology department of Saint Mary's Hospital and the original diagnoses were recorded and compared with new sections, as described in chapter 4.

7.1.2 Antibodies

Anti-bcl-2 oncoprotein monoclonal antibody (McAb, IgG 1) (human, clone 124) and anti-ras (pan clone F132-62) were purchased from Boehringer Mannheim Biochemica. The working dilution and concentration were set at 1:40 and 10 μg/ml respectively, by diluting in PBS (phosphate buffered saline), according to the manufacturer.

Anti retinoblastoma (Rb) gene protein McAb (IgG 1) and p53 McAb (IgG2b) were obtained from Novocastra Laboratories Ltd (24 Claremont Place, Newcastle Upon Tyne, NE2 4AA, UK) in lyophilised form. They were reconstituted with distilled water and adjusted to their working dilution at 1:20, as recommended by the producer.

Anti-PCNA (proliferative cell nuclear antigen) antibody was used to explore the proliferative status of tumour cells in comparison with those of normal counterparts.

The second (bridging) antibody, anti-mouse Ig-biotin (Sheep) was provided by Boehringer Mannheim Biochemica. It worked at a dilution of 1:500, as recommended by the manufacturer.

Other immunohistochemical staining regents were the same as those used in lectin staining procedures.

7.2 METHODS

7.2.1 Antigen retrieval procedures

Due to the rigours of routine fixation and processing protocols, the antigenicity of many proteins was either lost or masked. To unmask those antigens, Shi *et al* (1991) introduced the method of using microwave oven pre-treatment. This method was soon improved by Cattoretti *et al* (1992), in which they employed sodium citrate buffer instead. Microwave oven pre-treatment has been proven to be effective, but not efficient, because it is time-consuming.

Norton *et al* (1994) recently reported a method of unmasking antigens by using pressure cooking. It is very effective, and is also relatively simple, quick and convenient in allowing for the processing of batches of slides at one time. This method was used in this study. Its procedures are as follows.

1) Equipment: A stainless steel pressure cooker (Prestige, England) with capacity of around 5.5 litres, an electric plate or a gas ring.
2) Method:

Sections were mounted onto slides previously coated with 3-aminopropyltriethoxysilane (Apes) (Sigma Chemical Co.) in order to avoid the washing away of the sections. The slides were put into 56°C oven for drying. Prolonged drying (for instance, overnight) increased the attachment of sections onto the slides.

The sections were dewaxed in xylene and taken them to alcohol (99% v/v). Then block endogenous peroxidase with 0.5% (v/v) H_2O_2 in methanol for 10 minutes. Wash in water.

3 litres of 0.01M sodium citrate buffer, adjusted to pH 6.0 with HCl in the pressure cooker without pressure, was boiled. When the buffer was boiling, the slides were placed into the buffer and then covered with the lid. After the cooker came to the full pressure (120°C) and then boiled the slides for 1-2 minutes.

The cooker was cooled by quenching it in a sink of cold running tap water, removed the slides from cooker and quickly washed in running tap water, then transferred into Tris buffered saline (TBS). Immunostained the slides in the routine way.

7.2.2 Method of Immunohistochemistry

Avidin-biotin complex (ABC) was used in the following staining process.

- Pre-treated slides were washed in TBS (two changes), wiped out excess solution, apply each McAb at appropriate concentration onto the slides. Incubated in wet box 30 minutes at room temperature (18°C).
- Washed away the excess McAb then washed in TBS 3x5 minutes. Added sheep anti-mouse biotinylated IgG antibody and incubated 1 hour at room temperature(18°C).
- Washed the slides first with distilled water from a wash bottle then in 0.05M $CaCl_2$ TBS 3x5 minutes. Applied avidin-peroxidase (5μg/ml) and incubated at room temperature for 1 hour.
- After thoroughly wash the slides, visualisation is achieved by putting slides in DAB solution for 5 minutes.
- Counterstain the slides in 5% (v/v) methyl green 30 seconds. Rinse in tap water then dehydrate, clear and mount as usual.

7.2.3 Observation

All slides were observed under a light microscope (Leitz, Germany) and the recording was made based on the following standards: qualitatively: -, refers no staining or very faint positivity, +, refers to detectable positivity, ++, to clearly positivity, +++, to striking, intense positivity; quantitatively: counting 200 of positively stained cells in the densely populated positive foci and the numbers of positively stained cells among them, then calculating the percentages of positivity.

The positive cases were recorded according to the presence of positive staining, without considering the ratios of positive cells.

7.2.4 Analyses

The results of the expression of these oncogenes and tumour suppressor genes were compared with those of lectin histochemical staining to find if any relation exists between the expression of oncogenes and tumour suppressor genes and the alterations of gastric glycans as revealed by lectins. Student's test and Chi-square test were used, where appropriate, to analyse the data.

CHAPTER 8. RESULTS: EXPRESSION OF ONCOGENES AND TUMOUR SUPPRESSOR GENES

8.1 BCL-2 PROTEIN EXPRESSION IN GASTRIC TISSUES

Since this oncoprotein is the product encoded by the Bcl-2 oncogene (originating from B cell lymphoma) and is mainly distributed in the cytoplasm, it was readily seen in the B cells of the lymphoid component of the gastric mucosa (Figure 94). The B cells were intensely stained in their cytoplasm which outlined the cells clearly. Similarly, weak staining appeared in the gastric mucosal cells, normal or abnormal.

Generally, there was no stain or very weak staining in all layers of the normal gastric mucosae and the more strongly positive cells were mainly parietal cells, some of which showed a scattering of stained cytoplasmic granules. In tissues with chronic gastritis and intestinal metaplasia and/or dysplasia, positive staining could be seen in a small number of abnormal cells in 7/28, 6/26 and 3/10 cases, respectively.

In gastric tumours of all types, a proportion of cases showed positive staining with the percentage of cytoplasmic positive cells, ranging from 18% to 67% (Figures 95). Heterogeneous staining was observed in all types of gastric carcinomata and especially in the poorly-differentiated group. A great variation in the population of cells stained was seen among cases. The intestinal type of gastric carcinoma generally showed an increasing proportion of stained cells with decreasing differentiation. Simple diffuse carcinomata showed a few stained tumour cells and in 6/8 cases there was no stain at all. Although the average numbers of cytoplasmically positive cells appeared to be low, individual cases of some types

of gastric carcinoma had a high proportion of stained cells, reaching 67% in one case of the signet ring cell type (Figure 96). The detailed results are illustrated in table 8.1.

Table 8.1 The Bcl-2 expression in gastric carcinoma tissues

	Well	Moderately	Poorly	Mucinous	Signet
Positive cases	3/10 (30%)	4/11 (36.4%)	11/19 (57.9%)	2/8 (25%)	1/5 (20%)
Positive cells%	33.8±33.3	18.5±14.5	30.7±20.9	23.5±5.5	67

It is apparent from the table above that the intestinal-type of gastric carcinoma was more likely to express Bcl-2 oncoprotein (17/40, 42.5%) than the diffuse-type (3/13, 23.1%) ($p<0.001$). It also showed that low grade gastric carcinomata tended to express more Bcl-2 oncoprotein (poorly-differentiated carcinomata yielded about 57.9% of positive cases), while high-grade carcinomata showed fewer positive cases (30% and 36.4% in moderately- and well-differentiated carcinomata, respectively $p<0.01$). When the populations of positively stained cells were compared, there was a significant difference between the poorly-differentiated carcinomata and other types of gastric carcinomata ($P<0.001$).

Further comparison was made between the staining for Bcl-2 protein and the staining with those lectins which showed distinct staining in either normal or neoplastic tissues. An attempt was made to find any possible relation between the expression of Bcl-2 protein and the alterations in lectin-binding to gastric glycans. Since some lectins showed general staining on all of the tumour tissues, it was unnecessary further to analyse their relation to staining for Bcl-2 protein and other tumour suppressor gene and oncogene products. Hence, those lectins

are omitted hereafter. Table 8.2 is a summary of these comparisons.

Table 8.2 The comparisons of Bcl-2 expression and lectin staining

Lectin	Well-D		Moderately		Poorly-D		Mucinous		Signet	
	Bcl2+	Bcl2-	Bcl2+	Bcl2-	Bcl2+	Bcl2-	Bcl2+	Bcl2-	Bcl2+	Bcl2-
PSA +	2/3	1/3	2/5	3/5	4/8	4/8	2/4	2/4	1/3	2/3
-	1/4	3/4	0/1	1/1	2/6	4/6	0/2	2/2	0/2	2/2
LCA +	1/3	2/3	2/4	2/4	7/11	4/11	1/3	2/3	0/2	2/2
-	1/3	2/3	1/2	1/2	2/3	1/3	1/3	2/3	1/2	1/2
lPHA+	1/3	2/3	1/2	1/2	5/8	3/8	1/3	2/3	1/3	2/3
-	1/4	3/4	1/3	2/3	2/6	4/6	1/3	2/3	0/2	2/2
GNA +	2/3	1/3	2/4	2/4	4/8	4/8	2/3	1/3	1/2	1/2
	0/3	3/3	1/2	1/2	3/5	2/5	0/2	2/2	0/2	2/2
NPA +	2/3	1/3	2/3	1/3	4/7	3/7	1/2	1/2	1/1	0
-	0/4	4/4	1/3	2/3	2/6	4/6	1/4	3/4	0/4	4/4
UEA1+	3/9	6/9	4/8	4/8	9/12	3/12	1/3	2/3	0	0
-	0	0	0	1/1	1/3	2/3	1/3	2/3	1/4	3/4
LTA +	2/6	4/6	3/4	1/4	5/7	2/7	1/1	0	0	0
-	1/3	2/3	0/5	5/5	3/7	4/7	1/5	4/5	1/4	3/4
PNA +	3/3	0	3/4	1/4	9/13	4/13	2/6	4/6	1/3	2/3
-	0	6/6	1/5	4/5	2/5	3/5	0/2	2/2	0/2	2/2
BSA1+	0	0	2/5	3/5	2/6	4/6	1/3	2/3	0	0
$-B_4$ -	2/9	7/9	2/4	2/4	6/9	3/9	1/4	3/4	1/4	3/4
SNA +	2/6	4/6	2/6	4/6	9/16	7/16	1/6	5/6	1/3	2/3
-	1/3	2/3	2/4	2/4	1/3	2/3	1/2	1/2	0	1/1
MAA +	2/9	7/9	3/8	5/8	10/16	6/16	2/7	5/7	1/4	3/4
-	1/2	1/2	1/2	1/2	1/3	2/3	0/1	1/1	0/1	1/1
LFA +	0	6/6	2/5	3/5	4/8	4/8	1/4	3/4	0	0
-	2/3	1/3	1/3	2/3	4/6	2/6	1/4	3/4	1/4	3/4
VVA +	0/1	1/1	1/1	0/1	2/5	3/5	1/2	1/2	0/1	1/1
-	2/7	5/7	2/7	5/7	6/12	6/12	1/5	4/5	1/3	2/3
SBA +	2/7	5/7	3/6	3/6	6/9	3/9	1/3	2/3	1/2	1/2
-	0/2	2/2	1/3	2/3	2/6	4/6	1/4	3/4	0/3	3/3
PTA +	2/8	6/8	2/6	4/6	5/7	2/7	2/4	2/4	1/2	1/2
-	0	0	1/3	2/3	4/8	4/8	0/2	2/2	0/2	2/2
DBA +	0/2	2/2	0	0	1/4	3/4	1/2	1/2	0/1	1/1
-	2/8	6/8	3/9	6/9	8/13	5/13	1/5	4/5	1/3	2/3
LEA +	2/7	5/7	3/7	4/7	10/12	2/10	2/5	3/5	1/2	1/2
-	0	0	1/2	1/2	1/3	2/3	0/1	1/1	0/3	3/3
HPA +	3/8	5/8	3/8	5/8	9/14	5/14	2/6	4/6	1/4	3/4
-	0/2	2/2	0/2	2/2	2/4	2/4	0/2	2/2	0	0
CTA +	2/6	4/6	2/4	2/4	4/8	4/8	1/3	2/3	1/2	1/2
-	0/2	2/2	1/4	3/4	3/5	2/5	1/2	1/2	0/2	2/2
BSAII+	1/3	2/3	1/3	2/3	2/5	3/5	1/3	2/3	0	0
-	2/5	3/5	2/5	3/5	8/12	4/12	1/4	3/4	1/4	3/4

In most cases the alterations in glycans, revealed by lectin staining, had no evident correlation with the expression of Bcl-2 protein. However, some lectins did show such a relationship

with the staining for Bcl-2 protein, either in nearly all types of gastric carcinomata or only in certain subtypes. Those which showed significant statistical difference ($p<0.01$) included the following: PNA, MAA, SBA and HPA positive gastric carcinoma tissues of all types were more likely to show Bcl-2 expression; LCA, UEA-1, LTA and SNA positive cases also had a tendency to show elevated Bcl-2 staining (no statistical significance). An unusual phenomenon was that several lectins showed a negative relation with Bcl-2 staining, in which the 'lectin-positive' cases tended to show less expression of Bcl-2 and vice versa. Such lectins included BSA1-B_4, VVA and DBA. BSA-II.

8.2 RAS ONCOPROTEIN EXPRESSION

Normal gastric tissues showed no positive staining except for parietal cells, which had a granular cytoplasmic and membranous staining. In the precursor lesions, positive cytoplasmic and/or membrane staining was seen in chronic gastritic, metaplastic and dysplastic tissues of 4/29, 6/27 and 1/10 cases respectively, with differing proportions of positive cells.

Tumour tissues showed an elevated expression of *ras* oncoprotein in all types of gastric carcinomata (Figure 97, 98 99). Generally, the staining was mainly membrane-positive and intercellularly positive in the intestinal-type carcinomata, while in diffuse-type tumours, the staining was predominantly in cytoplasm. Signet ring cell tumours were different from the mucinous type in their components stained, in that the cytoplasmic staining was decreased or even negative. The results are summarised in table 8.3.

Table 8.3 The *ras* oncoprotein expression in gastric carcinomata

	Well	Moderate	Poorly	Mucinous	Signet
Positive cases	6/8 (75%)	6/8 (75%)	15/19 (78.9%)	5/8 (62.5%)	3/5 (60%)
Positive cells%	42.3±18.5	29.5±21.5	36.7±24.5	35.4±17.3	33.8±14.3

From the table above, it is evident that the expression of *ras* oncoproteins is common in gastric carcinomata. Intestinal-type tumours had a tendency to show more positive cases of staining for *ras* oncoprotein than did diffuse-type tumours. There was statistically a significant difference of *ras* protein expression between the intestinal-type and the diffuse-type of gastric carcinomata ($p<0.01$). In intestinal type, the well differentiated tumours had a higher proportion of positively stained cells (42%) than did the moderately differentiated carcinomata (30%) or the poorly differentiated tumours (37%). There were two cases of lymph node metastasis, which showed positive staining in almost all metastatic cancer cells.

A correlation between lectin stained cases and the expression of *ras* oncoproteins was made as above and is illustrated in table 8.4.

From the data in the table 8.4, it was found that there were several lectins which showed relations with the expression of *ras* oncoprotein. Grossly, there were three patterns of correlation.

In the first type it was found that staining for *ras* protein showed preferential expression in 'lectin-positive' tissues. Such lectins were PNA and MAA, in which a higher percentage of *ras* positive staining was observed in PNA or MAA positive cases than in negative ones ($p<0.01$). HPA also showed a similar relation to *ras* protein expression in all types of gastric carcinomata, except for the well-differentiated forms ($p<0.01$).

Table 8.4 The comparison of lectin staining with *ras* expression

Lectin	Well-D		Moderately		Poorly-D		Mucinous		Signet	
	ras+	*ras*-	*ras*+	*ras*-	*ras*+	*ras*-	*ras*+	*ras*-	*ras*+	*ras*-
PSA +	4/5	1/5	3/5	2/5	8/12	4/12	2/4	2/4	2/3	1/3
-	1/2	1/2	1/2	1/2	2/3	1/3	2/2	0/2	0/1	1/1
LCA +	2/3	1/3	3/4	1/4	8/11	3/11	2/3	1/3	1/3	2/3
-	2/4	2/4	1/2	1/2	2/3	1/3	2/3	1/3	2/2	0/2
lPHA+	3/3	0/3	2/3	1/3	5/8	3/8	2/3	1/3	2/3	1/3
-	3/4	1/4	2/3	1/3	4/6	2/6	2/3	1/3	1/2	1/2
GNA +	2/4	2/4	2/4	2/4	6/9	3/9	2/3	1/3	2/2	0/2
-	2/3	1/3	2/2	0/2	3/4	1/4	2/2	0/2	1/2	1/2
NPA +	2/3	1/3	2/3	1/3	5/7	2/7	2/2	0/2	1/1	0
-	3/4	1/4	2/3	1/3	4/6	2/6	2/3	1/3	2/4	2/4
UEA1+	6/8	2/8	5/7	2/7	12/14	2/14	2/3	1/3	0	0
-	0	0	1/1	0	2/4	2/4	3/4	1/4	3/5	2/5
LTA +	4/6	2/6	3/4	1/4	5/7	2/7	1/1	0	0	0
-	1/3	2/3	2/4	2/4	6/7	1/7	3/5	2/5	3/4	1/4
PNA +	3/3	0	3/4	1/4	11/13	2/13	4/6	2/6	2/3	1/3
-	2/4	2/4	1/4	3/4	3/5	2/5	1/2	1/2	1/2	1/2
BSA1+	0	0	2/4	2/4	2/6	4/6	1/3	2/3	0	0
$-B_4$ -	6/8	2/8	3/4	1/4	7/9	2/9	3/4	1/4	2/4	2/4
SNA +	2/5	3/5	2/4	2/4	13/16	2/16	3/6	3/6	2/3	1/3
-	3/3	0/3	3/4	1/4	2/3	1/3	2/2	0/2	1/2	1/2
MAA +	4/6	2/6	4/5	1/5	13/16	3/16	5/6	1/6	3/4	1/4
-	1/2	1/2	1/2	1/2	2/3	1/3	0/1	1/1	0	0
LFA +	0	0	3/5	2/5	5/8	3/8	3/4	1/4	0	0
-	4/6	2/6	2/3	1/3	5/6	1/6	2/4	2/4	2/4	2/4
VVA +	0/1	1/1	1/2	1/2	2/5	3/5	1/2	1/2	0/1	1/1
-	5/6	1/6	4/5	1/5	10/12	2/12	4/5	1/5	3/4	1/4
SBA +	4/6	2/6	4/6	2/6	7/9	2/9	2/3	1/3	2/2	0/2
-	2/2	0/2	2/2	0/2	4/6	2/6	2/4	2/4	1/3	2/3
PTA +	5/7	2/7	5/6	1/6	5/7	2/7	3/4	1/4	2/2	0/2
-	0	0	1/2	1/2	7/8	1/8	1/2	1/2	1/2	1/2
DBA +	1/2	1/2	0	0	2/4	2/4	1/2	1/2	1/1	0/1
-	4/5	1/5	6/8	2/8	11/13	2/13	3/5	2/5	2/3	1/3
LEA +	5/7	2/7	4/6	2/6	10/12	2/12	3/5	2/5	2/2	0/2
-	0	0	2/2	0/2	2/3	1/3	1/1	0/1	1/3	2/3
HPA +	4/6	2/6	5/6	1/6	12/14	2/14	3/5	2/5	3/4	1/4
-	2/2	0/2	1/2	1/2	2/4	2/4	1/2	1/2	0	0
CTA +	4/6	2/6	3/4	1/4	6/8	2/8	2/3	1/3	2/2	0/2
-	2/2	0/2	3/4	1/4	4/5	1/5	1/2	1/2	1/2	1/2
BSAII+	2/3	1/3	2/3	1/3	3/5	2/5	1/3	2/3	0	0
-	2/4	2/4	4/5	1/5	10/12	2/12	3/4	1/4	3/4	1/4

The second type of pattern was that a number of lectins showed a *limited* relation to the *ras* protein expression in *certain types* of carcinomata. Such lectins included PSA, LCA, UEA-1, SBA and LEA. PSA-positive tissues, including well- and moderately-differentiated carcinomata and signet ring cell tumours, showed a statistically significantly high expression

of *ras* oncoproteins. LCA-positive tissues showed elevated *ras* protein expression, mainly in the intestinal type of carcinoma rather than in the diffuse type. With UEA-1 and SBA, elevated staining for *ras* protein was limited to the poorly-differentiated gastric carcinomata. LEA-positive tumours showed enhanced *ras* protein expression both in poorly-differentiated and signet ring cell types.

The third sort of pattern was less clear-cut. It was that the tissues *negative* with lectin staining showed higher *ras* protein expression. Lectins seeming to show this behaviour included BSA1-B_4, VVA, DBA and BSA-II. Therefore, it is evident that there are links between alterations in glycan expression and the *ras* protein expression.

8.3 Rb PROTEIN EXPRESSION IN GASTRIC CARCINOMATA

Rb protein is a nuclear protein and this was reflected in the pattern of cellular staining for it, in which brownish granules were confined either within nuclei and/or along the nuclear membranes.

This onco-suppressor gene product showed no detectable expression in normal gastric epithelium (Figure 100), chronic gastritic, metaplastic (Figure 101) or dysplastic gastric tissues. However, the elevated expression of Rb protein did appeared frequently in several different types of gastric carcinomata, in which intense nuclear staining was observed in a variable proportion of tumour cells. It was also noticed that the intramucosal gastric carcinomata showed a large proportion cells with positively stained nuclei (Figure 102). A clear nuclear staining was shown in certain proportions of tumour cells as in Figures 103, 104. The proportions of positive cells showed variations in different types of gastric carcinomata and such variations were also observed within the same type of gastric

carcinomata, in which different cases gave various positive cells. Table 8.5 summarises the staining of Rb protein in all types of gastric carcinomata.

Table 8.5 The expression of Rb protein in gastric carcinomata

	Well	Moderately	Poorly	mucinous	Signet
Positive cases	4/9 (44.4%)	4/8 (50%)	11/19 (57.9%)	4/7 (57.1)	2/4 (50%)
Positive cells%	33.8±14.3	28.6±11.4	32.3±15.5	34.7±10.3	41.5±13.5

From the table above, it is apparent that Rb protein was expressed widely, but variably, in gastric carcinoma, ranging from 44% of cases in the well-differentiated type up to 58% of cases in the poorly differentiated group. It appeared that low-grade types of gastric carcinomata tended to express Rb protein, as it was detected in the intestinal poorly differentiated type (11/19 of cases) and in the diffuse type (57% of cases in the mucinous type and 2/4 cases of signet ring cell type), but there was no statistically significant difference found between them.

The proportions of cells positive for Rb protein expression increased from the well differentiated type towards tumours of morphologically lower degrees of differentiation, though statistically significant differences were not reached, except that the signet ring cell tumour which showed a significantly high proportion of stained cells than the others ($p<0.01$).

Hence, loss of Rb protein expression was not clearly related to the degree of tumour differentiation, but such a trend was noted and is possibly worth further study with larger series of tumours.

In order to determine if there is a correlation between Rb protein expression and lectin staining, a comparison was carried out, as before. This is illustrated in table 8.6.

Table 8.6 The relation between Rb expression and lectin staining

Lectin	Well-D		Moderately		Poorly-D		Mucinous		Signet	
	Rb +	Rb-	Rb+	Rb-	Rb+	Rb-	Rb+	Rb-	Rb+	Rb-
PSA +	3/5	2/5	3/5	2/5	7/12	5/12	2/4	2/4	2/3	1/3
-	1/3	2/3	1/1	0/1	2/3	1/3	2/2	0/2	0/1	1/1
LCA +	2/3	1/3	2/4	2/4	8/11	3/11	2/3	1/3	1/3	2/3
-	2/4	2/4	1/2	1/2	1/3	1/3	2/3	1/3	1/2	1/2
IPHA+	2/3	1/3	2/3	1/3	5/8	3/8	2/3	1/3	1/2	1/2
-	2/4	2/4	1/3	2/3	4/6	2/6	2/3	1/3	1/2	1/2
GNA +	2/4	2/4	2/4	2/4	6/9	3/9	2/3	1/3	2/2	0/2
-	2/3	1/3	1/2	1/2	3/4	1/4	1/2	1/2	0/2	2/2
NPA +	2/3	1/3	2/3	1/3	4/7	3/7	2/2	0/2	1/1	0
-	2/4	2/4	2/3	1/3	4/6	2/6	2/3	1/3	1/3	2/3
UEA1+	4/8	4/8	3/7	4/7	10/14	4/14	2/3	1/3	0	0
-	0	0	1/1	0	1/4	3/4	2/4	2/4	2/4	2/4
LTA +	3/6	3/6	2/4	2/4	4/7	3/7	1/1	0	0	0
-	1/2	1/2	1/2	1/2	5/7	2/7	3/5	2/5	2/4	2/4
PNA +	2/3	1/3	2/4	2/4	9/13	4/13	3/5	2/5	1/2	1/2
-	2/5	3/5	2/4	2/4	2/5	3/5	1/2	1/2	1/2	1/2
BSA1+	0	0	1/4	3/4	3/8	5/8	1/3	2/3	0	0
$-B_4$ -	4/8	4/8	3/4	1/4	7/9	2/9	3/4	1/4	2/4	2/4
SNA +	2/5	3/5	2/4	2/4	9/16	7/16	2/5	3/5	1/2	1/2
-	2/3	1/3	2/4	2/4	2/3	1/3	1/2	1/2	1/2	1/2
MAA+	3/6	3/6	3/5	2/5	9/15	6/15	3/6	3/6	2/4	2/4
-	1/2	1/2	1/2	1/2	1/3	2/3	1/1	0/1	0	0
LFA +	0	0	2/5	3/5	5/8	3/8	3/4	1/4	0	0
-	3/6	3/6	2/3	1/3	4/6	2/6	2/3	1/3	2/4	2/4
VVA +	0/1	1/1	1/2	1/2	1/5	4/5	0/2	2/2	0/1	1/1
-	3/6	3/6	3/6	3/6	9/12	3/12	4/5	1/5	2/3	1/3
SBA +	3/6	3/6	3/6	3/6	7/9	2/9	2/3	1/3	1/2	1/2
-	1/2	1/2	1/2	1/2	3/6	3/6	2/4	2/4	1/2	1/2
PTA +	3/7	4/7	3/6	3/6	5/7	2/7	3/4	1/4	1/2	1/2
-	0	0	0/2	2/2	5/8	3/8	1/2	1/2	1/2	1/2
DBA +	1/2	1/2	0	0	1/4	3/4	1/2	1/2	1/1	0/1
-	3/5	2/5	4/8	4/8	9/13	4/13	3/5	2/5	1/3	2/3
LEA +	3/7	4/7	3/6	3/6	9/12	3/12	3/5	2/5	1/2	1/2
-	0	0	1/2	1/2	1/3	2/3	1/1	0/1	1/3	2/3
HPA +	3/6	3/6	3/6	3/6	9/14	5/14	3/5	2/5	2/4	2/4
-	0/2	2/2	1/2	1/2	2/4	2/4	1/2	1/2	0	0
CTA +	3/6	3/6	2/4	2/4	5/8	3/8	2/3	1/3	2/2	0/2
-	1/2	1/2	2/4	2/4	3/5	2/5	1/2	1/2	1/2	1/2
BSAII+	1/3	2/3	2/3	1/3	2/5	3/5	1/3	2/3	0	0
-	2/4	2/4	2/5	3/5	8/12	4/12	3/4	1/4	2/4	2/4

When comparing the expression of Rb protein in the gastric carcinomata with the changes in glycan revealed by lectin staining, it was found that there were a number of lectin-positive gastric carcinoma tissues which showed preferential expression of Rb proteins ($p<0.01$). This preferential staining for Rb protein was observed in the following neoplastic tissues, as related to various lectins. With tissues stained with LCA and UEA-1, excess Rb protein expression was noted in poorly-differentiated carcinomata; with tissues stained with PSA, GNA and NPA in signet ring cell carcinomata; with tissues stained with PNA, SBA and DBA in well- and poorly-differentiated carcinomata as well as in mucinous tumours. MAA was found to be associated with Rb protein expression in moderately- and poorly-differentiated neoplasms as well as in signet ring cell carcinomata. HPA and Rb protein showed an overall correlation except in moderately-differentiated tumours.

Several other lectins, including BSA1-B_4, VVA and BSA-II tended to show underexpression of staining in cases positive for Rb protein expression.

8.4 P53 PROTEIN EXPRESSION IN GASTRIC TISSUES

In normal gastric tissues, one case was found to have recognisable nuclear staining in small clusters of epithelial and pit cells. No 'positive' cells were observed in either parietal cell or chief cell populations, nor in other stromal cells besides epithelial cells.

In chronic gastritic tissues, in contrast, two cases produced scattered positive cells in the pit area with whole nuclear staining or nuclear membrane staining (Figure 105)). Similarly, two cases of metaplasia showed nuclear staining in some cells. In the dysplastic lesions, 2 out of 5 cases expressed positivity for p53 protein in parts of the lesions.

Among the gastric carcinoma tissues, a high proportion of specimens showed intense nuclear positive staining in various percentages of tumour cells (Figure 107). Interestingly, one case of intramucosal gastric carcinoma showed a very high percentage (74%) of positive cells (Figure 106), which implies that abnormal expression of p53 can be a very early event in gastric carcinogenesis. The overexpression of p53 was much in evidence in the invasive margins of the tumour tissues (Figure 97). It was also found that p53 protein was expressed extensively in lymph node metastases. The p53 staining results are detailed as in table 8.7.

8.7 p53 expression in gastric carcinoma tissues

	Well	Moderately	Poorly	Mucinous	Signet
Positive cases	5/9 (55.5%)	5/8 (62.5%)	7/16 (43.8%)	6/8 (75%)	2/4 (50%)
Positive cells%	32.4±21.3	36.3±15.2	34.8±14.6	48.2±16.3	55.5±8.5

Based on the table above, it appeared that diminished differentiation in the intestinal type of gastric carcinoma tended to correlate with a decrease in expression of p53 protein. It was also noted that the diffuse type of gastric carcinoma, including mucinous and signet ring cell tumours, seemed more likely to express p53 protein in a considerable proportion of the tumour cells, as well as in more cases, than did the intestinal type of tumour.

The p53 expression of gastric carcinoma tissues was compared with the staining by those lectins which showed distinct patterns in the same set of tissues, in order to ask if there was a correlation between the elevated expression of p53 protein and the alterations in gastric glycans detected by those lectins. The results are summarised in table 8.8.

From the table 8.8, it is evident that elevated expression of p53 protein correlates with the staining by several lectins which displayed the alteration of glycans in gastric carcinoma tissues.

Table 8.8 The p53 expression and lectin staining

Lectin	Well-D		Moderately		Poorly-D		Mucinous		Signet	
	P53 +	P53-	P53+	P53-	P53+	P53-	P53+	P53-	P53+	p53-
PSA +	3/5	2/5	3/5	2/5	5/12	7/12	4/4	0/4	2/3	1/3
-	2/4	2/4	1/1	0/1	1/3	2/3	1/2	1/2	0/1	1/1
LCA +	2/3	1/3	3/4	1/4	5/11	6/11	2/3	1/3	1/2	2/2
-	1/4	3/4	1/2	1/2	1/3	2/3	2/3	1/3	1/2	1/2
l-PHA+	2/3	1/3	2/3	1/3	5/8	3/8	2/3	1/3	1/2	1/2
-	2/4	2/4	2/3	1/3	2/6	4/6	2/3	1/3	1/2	1/2
GNA +	2/4	2/4	3/4	1/4	5/9	4/9	2/3	1/3	2/2	0/2
-	2/3	1/3	1/2	1/2	2/4	2/4	1/2	1/2	0/2	2/2
NPA +	2/3	1/3	2/3	1/3	4/7	3/7	2/2	0/2	1/1	0
-	2/4	2/4	2/3	1/3	2/6	4/6	2/3	1/3	1/3	2/3
UEA1+	4/8	4/8	4/7	3/7	6/14	8/14	3/3	0/3	0	0
-	0	0	1/1	0	1/2	1/2	2/4	2/4	2/4	2/4
LTA +	4/6	2/6	3/4	1/4	4/7	3/7	1/1	0	0	0
-	1/2	1/2	1/2	1/2	2/7	5/7	3/5	2/5	2/4	2/4
PNA +	3/3	0/3	3/4	1/4	6/13	7/13	5/6	1/6	1/2	1/2
-	2/5	3/5	2/4	2/4	1/3	2/3	1/2	1/2	1/2	1/2
BSA1+	0	0	2/4	2/4	4/8	4/8	2/3	1/3	0	0
$-B_4$ -	5/8	3/8	3/4	1/4	3/7	5/7	4/4	0/4	2/4	2/4
SNA +	3/5	2/5	3/4	1/4	6/13	7/13	3/5	2/5	1/2	1/2
-	2/3	1/3	2/4	2/4	1/3	2/3	1/2	1/2	1/2	1/2
MAA+	4/6	2/6	4/6	2/6	6/12	6/12	4/6	2/6	2/4	2/4
-	1/2	1/2	1/2	1/2	1/3	2/3	1/1	0/1	0	0
LFA +	0	0	3/5	2/5	5/8	3/8	3/4	1/4	0	0
-	3/6	3/6	2/3	1/3	2/6	4/6	2/3	1/3	2/4	2/4
VVA+	1/1	0/1	2/2	0/2	3/5	2/5	2/2	0/2	0/1	1/1
-	3/6	3/6	3/6	3/6	4/12	8/12	4/5	1/5	2/3	1/3
SBA +	4/7	3/7	4/6	2/6	5/9	7/9	3/3	0/3	1/2	1/2
-	1/2	1/2	1/2	1/2	2/6	4/6	3/4	1/4	1/2	1/2
PTA +	4/7	3/7	4/6	2/6	5/7	2/7	3/4	1/4	1/2	1/2
-	0	0	1/2	1/2	2/8	6/8	2/2	0/2	1/2	1/2
DBA+	2/2	0/2	0	0	2/4	2/4	2/2	0/2	1/1	0/1
-	3/7	4/7	5/8	3/8	5/12	7/12	4/5	1/5	1/3	2/3
LEA +	4/7	3/7	4/6	2/6	6/12	6/12	3/5	2/5	1/2	1/2
-	0	0	1/2	1/2	1/3	2/3	1/1	0/1	1/2	1/2
HPA +	4/6	2/6	4/6	2/6	6/12	6/12	4/5	1/5	2/4	2/4
-	1/3	2/3	1/2	1/2	1/4	3/4	2/2	0/2	0	0
CTA +	3/6	3/6	3/4	1/4	5/8	3/8	3/3	0/3	1/2	1/2
-	2/2	0/2	2/4	2/4	2/7	5/7	2/2	0/2	1/2	1/2
BSAII+	2/3	1/3	2/3	1/3	3/5	2/5	2/3	1/3	0	0
-	3/5	2/5	3/5	2/5	4/11	7/11	3/4	1/4	2/4	2/4

Tissues positively stained with PNA, MAA and HPA generally showed an overall overexpression of p53 protein. An exception was noted in the mucinous type of carcinoma, which did not produce differences between MAA and HPA positive and negative cases. PSA, GNA and NPA staining showed a relation to the overexpression of p53 protein in low-grade carcinomata, including the poorly-differentiated and diffuse types, while LCA, VVA and LTA showed such a correlation with the intestinal type of gastric neoplasms. l-PHA, SNA, LFA, PTA and CTA showed a tendency to correlate with the elevation of p53 protein in poorly-differentiated carcinomata, though there was no statistical significance.

8.5 TUMOUR CELL LABELLING FOR PCNA

PCNA (proliferating cell nuclear antigen) reflects the cell proliferative activity of cells in cell cycle (late G1 and S phases) by showing an increase of PCNA in cell nuclei.

In normal circumstances (Figure 99), this protein is usually found in actively proliferative cells, such as the neck cells in gastric mucosa. Any factor which leads to cells to enter the cell cycle will increase the expression level of PCNA protein.

In normal gastric mucosa, PCNA expression was confined to the mucous neck cells, which showed a few cells with nuclear staining mainly scattered in the area between the surface epithelial cells and gland cells (Figure 108). The staining was seen as light brownish granules in the nuclei of neck cells.

In the precursor lesions of gastric carcinoma, the proportion of stained cells was increased and was found in the actively proliferating areas, corresponding to the normal

neck zone, which led to the elongation of the neck area. In this zone, a large number of cells were intensely stained showing dark brownish nuclei in arrays of columns or clusters. Gastritic tissues generally showed an increased number of PCNA-positive staining cells in the elongated neck zones (Figure 109). Metaplastic tissues had a similar staining pattern and the goblet cells did not show positive staining, while dysplastic tissues yielded clustered positive cells at the affected glands and their staining was very dense.

Tumour tissues and cells showed variable staining, both in the proportion of the PCNA-positive cells and their staining intensity. Grossly, the well- and moderately-differentiated gastric carcinomata showed a small percentage of cells with positive nuclei dotted about in the tumourous glandular structures. Staining was in the form of light brownish nuclear granules (Figure 110). In contrast, the staining of low-grade carcinomata, such as poorly-differentiated and mucinous tumours, showed a high proportion of tumour cells with nuclear staining. It was also noted that the staining became much more prominent in the invading margins of the gastric carcinomata, suggesting the active growth of tumour cells in the outer parts of tumours.

Mucinous and signet ring cell carcinomata also showed a high percentage of positive cells which were associated with the mucin pools (Figure 111). The staining results for PCNA protein are outlined in table 8.9.

Table 8.9 The expression of PCNA in gastric tumours

	Well (9)	Moderately (8)	Poorly (17)	Mucinous (7)	Signet (5)
+cells%	23.3±15.8	26.3±18.2	34.8±24.6	45.2±17.3	52.5±18.5

It is evident from the table 8.9, that the expression of PCNA protein increased with the anaplasia of tumours and atypia of the tumour cells. This was reflected in the increasing populations of positive tumour cells from well-differentiated to lower-grade gastric carcinomata. There were statistically significant differences between the intestinal-type and the diffuse-type carcinomata ($p<0.01$). It was evident that the proportions of PCNA-positive cells increased from the higher grades of tumours, as in well-differentiated carcinomata, to lower grade as in poorly-differentiated and diffuse tumours .

The correlation between the expression of PCNA and the lectin staining was investigated. The results are recorded in table 8.10.

The data in table 8.10 showed that the alterations of gastric glycans shown by several lectins could have a relation to the expression of PCNA protein, in that the elevated expression of PCNA was much more common in those tissues stained strongly by some lectins, while the converse was rare.

All types of gastric carcinomata showed an overall increased PCNA expression in some lectin-positive tissues rather than in the lectin-negative ones. Lectins showing this phenomenon included PNA, MAA, SBA and HPA, although variations existed in subtypes. UEA-1 and LFA also showed a similar tendency. Some types of carcinomata give more expression of PCNA in lectin-positive tumour tissues than in negative ones. These lectins were PSA and GNA in intestinal-type carcinomata and NPA, l-PHA, LTA and SNA in low-grade carcinomata. The elevated expression of PCNA was more frequent in lectin-negative tissues only with BSA1-B_4 and DBA.

Table 8.10 The expression of PCNA and the lectin staining

Lectin	Well-D	Moderately	Poorly-D	Mucinous	Signet
PSA +	27.4±12.3	28.5±14.3	43.3±11.7	50.3±16.4	54.3±13.5
-	22.5±8.5	23	24.6±7.3	41.5±14.5	-
LCA +	19.5±10.5	28.5±9.5	37.3±25.43	38.6±18.1	55.5±13.562
-	31.3±12.6	23.5±20.5	3.5±17.5	48.3±14.4	
l-PHA+	19.6±13.4	31.4±12.6	44.2±20.33	54.3±16.3	60.3±23.4
-	28.7±20.3	25.5±14.3	5.3±13.2	39.5±15.4	-
GNA +	34.2±16.4	41.4±19.2	40.6±22.43	42.2±18.1	64.5±22.551
-	19.3±8.6	21.5±10.5	2.3±15.2	51.5±16.5	
NPA +	21.6±11.3	33.6±15.3	45.2±23.43	53.5±15.5	51
-	29.5±14.5	25.4±12.4	3.6±14.4	51.6±17.3	54.5±20.5
UEA-1+	23.3±15.8	28.3±24.6	36.7±25.33	52.4±17.4	-
-	-	22	3.5±16.4	46.5±20.5	52.5±18.5
LTA +	25.4±16.3	30.1±22.4	40.6±23.83	55	-
-	-	24.3±15.6	6.2±19.1	43.7±20.3	52.5±18.5
PNA +	35.3±12.4	30.2±15.4	38.3±21.43	50.2±21.4	61.3±17.247.5±
-	21.4±9.3	25.3±17.4	2.3±16.3	31	21.3
BSA1+	-	23.4±16.3	31.5±18.43	38.4±15.2	-
$-B_4$ -	24.6±16.3	33.2±20.4	9.3±13.4	50.6±17.2	55.6±20.1
SNA +	27.4±14.3	25.8±14.7	36.1±18.43	51.2±20.3	48.8±19.461
-	21.5±11.3	30.2±13.6	1.6±16.2	31	
MAA+	26.2±14.6	31.3±16.4	37.7±26.32	46.2±15.3	57.3±20.443
-	18.5±9.3	20.5±11.5	9.4±17.3	44	
LFA +	-	34.2±19.3	38.2±16.43	50.3±21.5	-
-	25.2±13.6	24.5±12.5	2.7±19.2	38.4±18.6	55.6±18.4
VVA +	20.5±11.5	22.5±13.5	30.6±18.34	49.5±27.5	30
-	28.6±16.3	33.4±17.4	1.2±25.4	44.2±16.2	62.4±20.3
SBA +	27.2±18.3	35.6±20.3	42.8±21.6	49.3±23.4	45.7±22.658.4±
-	19.4±9.6	21.5±13.7	33.4±15.3	44.4±17.3	17.5
PTA +	24.7±13.6	25.4±15.3	32.8±21.54	38.8±16.3	43.6±14.757.3±
-	-	29.6±17.2	0.3±23.3	51.5±14.5	12.5
DBA +	18.5±7.4	-	31.4±15.33	37.5±13.5	37
-	29.6±16.5	26.3±18.2	8.7±17.4	54.3±21.4	61.4±16.8
LEA +	23.3±15.8	40.2±21.8	32.6±20.44	50.4±14.4	43.5±16.359.3±
-	-	21.5±9.5	1.3±15.2	38	18.4
HPA +	33.5±14.7	35.1±16.3	40.6±17.22	51.7±13.4	58.3±16.447
-	19.4±8.3	16.5±7.2	9.4±14.3	40.5±17.4	
CTA +	27.4±13.8	29.4±16.2	33.8±19.34	37.3±17.2	60.5±16.554.3±
-	20.5±6.5	24.6±14.5	1.3±11.7	62.5±13.3	12.6
BSAII+	22.5±8.3	31.3±14.6	40.3±21.73	44.5±23.6	-
-	27.9±14.2	26.7±11.6	3.6±16.4	51.3±14.3	56.2±16.4

CHAPTER 9 DISCUSSION

9.1 THE EXPRESSION OF BCL-2 PROTEIN IN GASTRIC TISSUES

Previous studies have revealed that Bcl-2 may be involved in gastric carcinogenesis (Lauwers *et al*, 1994), especially in the low-grade type of gastric carcinoma (Ayhan *et al*, 1994). In this study, it was found that this oncogene was expressed frequently in precancerous gastric lesions as well as in some cases of gastric carcinoma.

Bcl-2 protein was expressed in one-fourth of cases of gastritis and metaplasias, and one-third of dysplasias, showing an increasing tendency with anaplasia of the affected epithelia. Most positive cells were localised in areas of active cellular proliferation. It was also noted that the expression of Bcl-2 protein tended to increase in cases from well-differentiated to poorly-differentiated carcinomata. These findings suggest that the Bcl-2 gene starts to function in the early stages of gastric carcinogenesis by impeding cell apoptosis and, consequently, prolonging cellular life span, so increasing the risk of activating other oncogenes, and thereby leading to an increased probability of tumourigenesis. This is most likely to apply to the intestinal type of gastric neoplasm, since a linear increase of expression of Bcl-2 protein was seen from gastritis, metaplasia to dysplasia, and from high grade to poorly-differentiated carcinoma. The gene generally persists in its expression after the appearance of a frank carcinoma, so that its oncoprotein continues to be expressed in an increasing proportion of neoplastic cells, especially in the intestinal type of gastric carcinoma.

In contrast, it was found that Bcl-2 protein was less frequently expressed in the diffuse type of gastric carcinoma and that the difference was significant. Furthermore, the metastases did not

show a detectable expression of Bcl-2 protein. This may imply that Bcl-2 is involved in the onset and subsequent development of these gastric neoplasms, but with tumour progression, this gene may be either switched off or suppressed by other factors. Alternately, the gene product may become subject to increased degradation or sequestration.

This study also found that the elevated expression of Bcl-2 protein had significant associations with alterations in some types of glycans detected by a number of lectins in the tumour tissues. This has not previously been described.

Several types of sugar sequences appeared to be more abundant in those gastric carcinoma tissues which had an increased expression of Bcl-2 protein. Such sugar residues appeared to include: Gal β1,3 GalNAc α1- (detected by PNA), NeuNAc α2,3 Gal β1- (detected by MAA), terminal GalNAc α1- (bound by SBA) and D-GalNAc (bound by HPA). Some other sugar sequences also showed an increased trend (below statistical significance) in the tumour tissues with elevated expression of Bcl-2 protein. These included those revealed by LCA, UEA-1, LTA and SNA.

In contrast, some structures were diminished or lost in those tumour tissues expressing Bcl-2 protein. Those with a statistically significant association putatively included: Gal α1,3 Gal β1,4 GlcNAc β1- (shown by $BSA1B_4$), GalNAc α1,3 Gal β1 (bound by VVA), GalNAc α1,3(Fuc α1,2) Gal β1,3/4 GlcNAc β1 (shown by DBA) and D-GlcNAc (bound by BSAII). The mechanisms responsible for these associations are not clear, but several may be postulated.

One possibility is that the overexpressed Bcl-2 protein is itself glycosylated by those specific

sugar sequences that are increased. There is no report of the glycosylation of Bcl-2 protein, but the glycosylation of c-myc has been recently reported (Chou *et al*, 1995). Evidence was found for O-linked glycosylation of c-myc oncoprotein with N-acetylglucosamine, a helix-loop-helix leucine zipper phosphoprotein, which can form a heterodimer with Max and participate in the regulation of gene transcription in normal and neoplastic cells. This glycosylation of c-Myc was shown by using lectin binding to the c-myc translated protein *in vitro* and glycosidase or glycosyltransferase treatment of the c-myc translated protein. Chou *et al* also suggested that the O-GlcNAc site(s) of c-myc protein were located within or near the N-terminal malignant transformation domain. It may be possible that Bcl-2 undergoes a similar process of modification.

Secondly, the syntheses of mucin core proteins and their subsequent glycosylation with some types of glycans could be enhanced while the synthesis of other types of glycans could be either suppressed or completely switched off. This might be caused by the increase or decrease of the appropriate glycosyltransferase activities during carcinogenesis and/or development. Previous reports have found elevated or depressed activities of some glycosyltransferases, mainly related to blood group substances, in tumour cells and tissues (Dohi *et al*, 1994; King *et al*, 1994). Dohi *et al* (1994) found that sialyltransferase activity for sialyl Le^a was shown in most normal or malignant gastric mucosae, while the transferase activity for sialyl Le^x showed low activity in normal gastric mucosa, but the sialyl Le^x transferase was significantly increased over normal in 77% of gastric neoplasms than in normal tissues. King *et al* (1994) found that a defect in glycosyl transferase of O-linked oligosaccharides led to an abnormal staining for α-N-acetylgalactosamine-O-Ser/Thr (Tn antigen) and sialyl-Tn in colorectal carcinoma cells. An increase in supranuclear staining over controls was found in the tumour tissues and in the majority

of specimens of resection margin, in contrast to its being weak in normal colorectal cells and absent in the normal goblet cell vesicles. The highest levels of staining were present in transitional mucosa, adjacent to the tumours, in which goblet cells were stained. The activity of glycosyltransferases in mucosal tissue from controls and tumours were further determined and the reduction in, or loss of, β1,3 N-acetylglucosaminyl transferase activity was found in tumour tissues. These studies imply that changes of glycosyl transferases underlie the alterations in gastric glycans.

Another, third, possibility is that there was no change in the amount of particular sugar sequences in the glycans of the tumour tissues, but that their configurations were altered during tumourigenesis, leading to the exposure of some sugar sequences, which became more accessible to lectin binding, while others were blocked and so were not easily demonstrated by lectin staining. Gastric glycans are macromolecules with a high molecular weight and they contain long and branched sugar chains, which have great configurational flexibility. Consequently, they have very a complicated stereochemistry, in which some sugar residues are well exposed while others may be occluded partly or completely. In abnormal conditions such as tumourigenesis, this abnormality may become evident.

Finally, it is also possible that all three potential mechanisms contribute to the associations between the expression of Bcl-2 protein and alteration of gastric glycans.

9.2 THE EXPRESSION OF RAS ONCOPROTEIN IN GASTRIC TISSUES

Since there was no detectable positive staining in normal gastric epithelial cells, it implies that this gene is rarely activated in the normal state, and this is consistent with the findings of others (Czerniak *et al*, 1989; Nakajima *et al*, 1989; Carneiro *et al*, 1992; Teh and Lee, 1993).

In the 'precancerous' gastric lesions, the expression of the oncoprotein p21 encoded by *ras* oncogene was often seen in varying proportions of cells in gastritic, metaplastic and dysplastic tissues. Those cases with expression of p21 tended to increase in frequency with the severity of the mucosal lesions. This suggests that the activation of *ras* oncogene is early and may occur close to, or even be responsible for, the initiation event, though it could also be involved in the later progression of gastric carcinomata.

From the results above, it is evident that a high proportion of p21 expression occurred during the development of both types of gastric carcinoma, and that this is reflected in the numbers of 'positive' cases as well as in the proportions of 'positive' cells. It was evident that *ras* oncogene and its product continued to be expressed after the onset of gastric carcinoma. It was further noted that there were no significant differences either between the intestinal type and the diffuse type of carcinoma or within the intestinal subtypes of gastric neoplasms. This may imply that the activation of *ras* gene and the function of p21 were equally important in the carcinogenesis and progression of both types of gastric carcinomata.

The strong expression of *ras* oncogene product p21 in the metastases was notable; all such secondary tumours showed positive staining with a high proportion of tumour cells being positive.

In summary, *ras* oncogene and its p21 oncoprotein probably play a very important role at an early stage of gastric carcinogenesis, as indicated by previous work (Nakajima *et al*, 1989; Carneiro *et al*, 1992; Teh and Lee, 1993) and they may also be important in later tumour progression, although the detailed mechanisms involved remain unclear.

In correlating the staining of lectins with the expression of p21 protein, certain saccharide sequences were found to have a relation to the elevated level of detectable p21.

It was evident that some sugar residues were stained more strongly by some lectins in the *ras* p21-positive cases while others diminished or completely disappeared. The sugar sequences showing an overall increase in the p21-positive tumour tissues included: Gal β1,3 GalNAc α1- (shown by PNA), NeuNAc α2,3 Gal β1- (detected by MAA) and D-GalNAc (shown by HPA). Some sugar sequences or structures showed increases only in a certain types of gastric carcinoma, such as α-mannose in non-bisected bi/tri-antennary N-linked sequences by PSA and LCA in the intestinal type of carcinoma. Others included the sequences detected by SBA in high grade neoplasms and LEA in most types of tumour except for moderately-differentiated and diffuse carcinomata .

The sugar residues decreased in *ras*-positive cases were very similar to those diminished in cases positive for Bcl-2 protein. These were, tentatively, Gal α1,3 Gal β1,4 GlcNAc β1- (shown by BSA1-B_4), GalNAc α1,3 Gal β1- (shown by VVA), GalNAc α1,3(Fuc α1,2) Gal β1,3/4 GlcNAc β1- (bound by DBA) and D-GlcNAc (detected by BSAII).

Again, the mechanisms involved were not clear and could be of the same general types as the postulations of those mechanisms suggested for Bcl-2 above.

It has not been reported if the post-translational modifications of *ras* p21 protein are involved in glycosylation of the protein. If such a modification occurs, it may be responsible for the altered expression of specific sugar sequences of glycans, partly or totally.

Elevated activities of glycosyltransferases have been documented in the literature (Easton *et al*, 1991; Wojciechowicz *et al*, 1995), and certain sugar sequences have been related to the function of *ras* p21 proteins. In the study of Wojciechowicz *et al* (1995), it was found that five out of six cell lines with K- *ras* mutations showed elevated amounts of beta 1-6 branching at the trimannosyl core of N-linked carbohydrate, while no elevated beta 1-6 branching was seen in K- *ras* wild type cell lines. The amount of beta 1-6 branching correlated with the extent of cellular *ras*-GTP elevation and supports the hypothesis that expression of beta 1-6 branching in colon carcinoma cell lines is quantitatively linked to K- *ras* activation.

In another report by Easton *et al* (1991), the changes in various glycosyl transferases consequent upon the activation of *ras* gene were analysed. Their results showed that the levels of N-acetylglucosaminyltransferases I and II were not changed, suggesting that the basic synthesis of complex-type glycans was unaffected; but N-acetylglucosaminyltransferases III and V were elevated 2- to 2.5-fold, indicating a potential increase in formation of bisected glycans and of structures with a Gal β1,3 GlcNAc β1,6 Man-branch; the increase of β4-galactosyltransferase and β3-N-acetylglucosaminyltransferase was most evident (5- to 7-fold), indicating a strongly enhanced capacity to synthesise polylactosaminoglycan chains. It was also found, in this study, that the level of the major chain- terminating enzyme, alpha 3-galactosyltransferase, was slightly decreased (0.7 fold), whereas those of the alpha 3- and alpha 6-sialyltransferases were slightly elevated (1.3- and 2-fold, respectively), suggesting a shift from termination by alpha-galactosyl residues to termination by sialyl moieties. This is consistent with the finding in this study that MAA staining was enhanced in *ras* positive cases.

Analysis of the size of cell surface complex-type glycopeptides before and after digestion with neuraminidase and endo-β-galactosidase suggested an increased sialic acid density, an increase in the number and/or length of polylactosaminoglycan chains and an increased branching of the glycans upon N- *ras* induction. The changes in enzyme activities found by Easton *et al* (1991) explain these structural changes and illuminate the alterations in glycosylation pathways associated with *ras* expression.

It is, therefore, reasonable to propose that changes in glycosyl transferases, consequent upon the activation of *ras* oncogene play very important roles in generating the alterations in gastric glycans associated with neoplasms.

9.3 THE RB ONCOGENE AND ITS ONCOPROTEIN

The retinoblastoma (Rb) gene is one of the best characterised tumour suppressor genes. It is located on the chromosome 13q14, was the first tumour suppressor gene identified and serves as the prototype for its behaviour. Its oncoprotein, Rb protein, has a known structure of 928 amino acids and a molecular weight of 110 kDa. The p110 Rb protein plays an important role in cell proliferation, and differentiation, as well as in carcinogenesis.

This protein can exist in the nucleus in both hyper- and hypo- phosphorylated forms, the ratios of which vary at different stages of the cell cycle. The hypophosphorylated Rb protein appears through most of G1, while the hyperphosphorylated form is found in the late G1, S, G2 and M phases. This suggests that Rb protein functions in regulating the cell cycle through a phosphorylation mechanism which switches at the beginning and near the end of the G1 phase.

Experiments have shown that the hypophosphorylated Rb protein can inhibit cell cycle progression and leads to arrest of the cell cycle in G1 phase (Cobrinik *et al*, 1992 Livingston *et al*, 1993).

Normally, during the G1 phase, cells respond to extracellular signals by either advancing toward another division, through the S, G2, and M phases, or withdrawing from the cycle into a resting state (G0 phase). Most oncogenic proteins exert their effect by targeting particular regulators of G1 phase and lead to cellular progression (Hall and Peters,1996). The hypophosphorylated Rb protein inhibits cell cycle progression by binding to and inactivating a set of cellular transcription factors, such as E2F and Elf-1 and results in the arrest of cell cycle progression at G1 phase. After cell activation, Rb is rapidly phosphorylated and the Rb-E2F and Rb-Elf-1 complexes are thereby disrupted. The release of these transcription factors leads to cellular progression through the G1 and over the S checkpoint of the cell cycle, so leading to subsequent cellular proliferation.

Other study also provides strong support for the role of Rb protein in G1 arrest (Hinds and Weinberg, 1994). The transition between the different forms of Rb protein reflects its state of activity. The hypophosphorylated Rb protein is an activated form, which is responsible for its normal function in binding to cellular transcription factors and thereby inhibiting their cell cycle-stimulating effects, while the further phosphorylation of Rb protein causes its functional inactivation. This phosphorylation process is now believed to relate to the cyclins (cyclin A and E, for example) which regulate the specificity and activity of cyclin-dependent kinases (cdk). Evidence has been obtained *in vitro* that the cyclins A and E are critical for the hyperphosphorylation of the Rb protein in late G1 phase (Ewen, 1994).

The function of Rb protein is achieved through its binding to nuclei by way of its combining with its nuclear partner protein or proteins (collectively termed the 'nuclear anchor'). Thus, any factors which affect this binding, or the structure of Rb protein, and especially those which influence crucial binding sites, can lead to a failure of the protein to arrest the cell cycle, so causing unregulated division, cellular 'overgrowth', diminished apoptosis and a risk of malignancy. It has been found that some viral proteins, such as adenovirus E1A, can bind to Rb protein (Lukas *et al*, 1994). It may be inferred that the competitive binding of Rb protein by proteins other than its nuclear anchor, and chromosomal mutation, can both cause the loss of the cycle-suppression function of Rb protein.

Rb protein is expressed in various types of cells, but most of them are proliferating and postmitotic cells (i.e. cells in cell cycle), while quiescent cells which have left the cycle seldom show a detectable content of this protein. Hence, it appears that Rb protein is expressed by cells undergoing differentiation to produce non-dividing end cells or offspring cells that are different from their parent cells and are reversibly outside the cell cycle.

Abnormal expression of Rb occurs in a wide range of human tumours. However, there is no report of this oncogene and its protein product in relation to gastric tissues. It was, therefore, reasonable to carry out such an investigation, to determine whether this gene is involved in gastric carcinogenesis.

In this study, it was found that there was no detectable expression of Rb oncoprotein in normal gastric tissues and the precursor lesions of gastric neoplasms. However, it was found to be expressed in 24/35 of cases of overt malignant neoplasia, though the proportions of positive cells varied according to the subtype of carcinoma.

In the intestinal type of gastric carcinoma, there was a tendency for an increased expression of Rb protein with increasing anaplasia. It was found that the 'positive' rates rose from 40% and 33% to 79% in well, moderately and poorly differentiated carcinomata, respectively. There was a significant difference between the poorly differentiated type and the other two intestinal types of gastric carcinoma ($p<0.001$). The proportions of 'positive' cells in each subtypes showed no obvious variations and were less than one-third of all the tumour cells. Furthermore, the 'positive' cells were weakly stained in all cases. This implies that the expression of Rb protein was only moderately increased.

In the diffuse type of gastric carcinoma, 66% of cases showed positive expression of Rb protein (this included two positive cases of signet ring cell type). The positive cells were present in statistically significant higher numbers than were those in the intestinal type ($P<0.001$). It was also noted that all the metastatic carcinomata, as well as intramucosal carcinoma, showed expression of Rb protein.

From the results above, it can be postulated that the Rb protein expressed in gastric carcinomata may be a mutated form of normal Rb protein and that the mutation may occur at an early stage of gastric carcinogenesis, as found in the intramucosal carcinoma. The mechanisms involved are not known. In other words, it can be hypothesised that the expression of normal Rb protein was suppressed in the 'precursor' lesions. During transformation, in early carcinogenesis, a change occurred in the 'pre-neoplastic' cells which resulted both in the release from inhibition of an Rb gene and a mutation, such that it caused synthesis of mutant Rb protein, detected as excess Rb protein in the tumours. This expression of abnormal Rb protein persisted and possibly became more marked during the

development and progression of the tumours, possibly as a reactive, compensating measure, since the mutated Rb protein may not have been competent to perform its normal suppressive function in restraining the growth of the incipient tumour cells. This is consistent with the higher 'positive' cell fraction seen in poorly differentiated and diffuse types of gastric carcinomata.

Another possibility is that the appearance and subsequent elevated level of expression of Rb protein was the result of its sequestration as *normal* Rb protein by some other mutated proteins, such as parts of the anchor.

Little is known about the expression of Rb protein in gastric neoplasms, although it was noted that in gastric and colorectal cancer, Rb gene loss or deletion was a rare event (Monden *et al*, 1996). Ogawa *et al* (1996) detected Rb protein in 48/94 gastric tumours (51.1%). They did not find significant association of the expression of Rb with histologic type, lymph node or liver metastasis. However, a significant relation was noted with respect to depth of invasion and peritoneal metastases. That is, less Rb expression was detected in deeper invasion and metastasis. In relation to prognosis, patients with Rb-positive tumours had a significantly better prognosis than those with Rb-negative carcinomata. They suggested that expression of Rb has a prognostic value in gastric cancer. However, more work is needed fully to understand the role(s) of Rb protein in gastric carcinogenesis.

Comparing the changes in glycan expression, as shown by lectin binding, with the expression of Rb protein, it appeared that the overexpression of Rb protein was generally related to an increase in specific sugar sequences in subtypes of carcinoma. Sugar sequences which increased with high Rb expression included α-mannose in non-bisected bi and tri-antennary

N-linked chains (as shown by LCA) and L-Fuc α1,2 Gal β1,4 GlcNAc β1- (as detected by UEA-1) in poorly-differentiated carcinomata; and α-D-mannose, with α1,3 or α1,6 linkages (bound by GNA and NPA, respectively), in signet ring cell tumours. Some glycans appeared more likely to increase in most types of carcinomata with elevated Rb expression, such as those revealed by PNA, SBA, DBA and MAA, in which only one or two subtypes of carcinoma failed to show correlation with the expression of Rb protein. Other glycans decreased in the Rb-positive tissues were also detected by $BSA1B_4$, VVA and BSAII.

There is no previous report of any relation between alterations of glycans and the overexpression of Rb protein in gastric carcinomata. Similar causative mechanisms to those for Bcl-2 and *ras* may be postulated.

There is no publication concerning the glycosylation as one of the post-translational modifications and such event might occur. The elevated expression of Rb protein, either mutated or sequestered 'normal' Rb protein seen in the gastric carcinoma, might provide conditions for the abnormal glycosylation of Rb protein. However, if this is the case, Rb protein can not be attributable for the overall increased alteration of glycans, since it is present only in nuclei. This postulation remains uncertain and doubtful.

Changes in glycosyl transferases are a more plausible explanation. Since the overexpression of Rb protein occurred in the early stage of gastric carcinogenesis and persisted into the late progression, it might affect the syntheses of certain types of sugar chains by altering the activities of glycosyltransferases by Rb protein itself or under the influence of other factors. If such events occur, the amount of the sugar residues detected by lectins would fluctuate during the tumour progression. The alteration in gastric glycans might not have any direct

relation with the elevated Rb expression, but only reflect the alterations of other factors, which have more influence over the synthetic process of sugar chains. Therefore, their relation in the gastric carcinogenesis needs further clarification.

9.4 P53 EXPRESSION IN GASTRIC CARCINOGENESIS

P53 tumour suppressor gene, though active, only leads to a low expression of its p53 protein in normal gastric tissues and only one case has showed scattered positive cells in the superficial layer of the mucosa. This is in contrast to its higher expression in chronic gastritis and dysplasia with 2/11 and 2/5 of positive cases, respectively.

The slight staining in normal tissue may represent the usual function of the normal p53 gene. The elevated proportions of positive cells in these precancerous lesions, on the other hand, suggest either that the normal P53 gene was activated in response to external stimuli and so an increased amount of p53 protein was expressed, or that the P53 gene has undergo mutation and consequently expressed mutated p53 protein which was not degraded normally. This can be considered as an ineffectual response against abnormal proliferation. That is, in an inflammatory or a reactive condition, P53 gene is likely to be expressed. It may later become mutated, which results in the excessive expression of p53 protein in those inflammatory lesions.

In gastric carcinoma tissues, p53 protein was expressed in over half of the cases (9/15), with a high proportion of 'positive' tumour cells. There was no significant difference between the intestinal and diffuse types of gastric carcinoma. It was noted that one case of intramucosal carcinoma strongly expressed p53 protein with 75% of tumour cells being positive. This gives

direct evidence to support the hypothesis that p53 mutation is an early event in gastric carcinogenesis.

Previous studies on this gene and its products in gastric carcinoma have drawn similar conclusions to those from this study. As in one report by Shiao *et al* (1994), it was found that alterations of the p53 gene occurred in an increasing order of 'positivity' from metaplasia, through dysplasia, to carcinoma, suggesting that the p53 gene mutation is one of the earlier events in gastric carcinogenesis. Evidence from other studies also implies that p53 gene deletion and mutation are common events in gastric carcinogenesis (Matozaki *et al*, 1992; Seruca *et al*, 1992; Renault *et al*, 1993). Fukunaga *et al* (1994) in their immunohistochemical study, found that p53 protein was expressed significantly more highly in cases of intestinal gastric carcinoma than in the diffuse type. They suggested that the p53 gene mutation was a common event and might participate in the carcinogenesis of the intestinal type carcinoma of the stomach rather than in that of the diffuse type.

One previous report has assessed the changes in glycan detected by HPA in relation to the expression of p53 protein in gastric carcinoma (Maehara *et al*, 1995). It was found that the change in a glycoprotein defined by the binding of *Helix pomatia* agglutinin (HPA) identified a subset of cases with a higher 'positive' rate of abnormal p53 staining and a higher concentration of proliferating cell nuclear antigen (PCNA) than the remainder. They also observed that the survival time of the patients with HPA-positive staining was shorter than for those whose tissues were HPA-negative. It was concluded that a tumour-associated cell-surface glycoprotein, containing HPA-specific glycan sequences, was closely related to the malignant potential of serosally invasive gastric cancer.

In this study, similar results were found in relation to the HPA-binding glycan which was detected more commonly in the p53-positive tumours than in those which were p53-negative. It was also noted that there was no significant difference of HPA staining in the diffuse type of gastric carcinomata with or without p53 overexpression, since p53 presented an overall positive staining in all cases of tumours of this type.

Other sugar sequences generally increased with elevated p53 expression were those bound by PNA and MAA. Alterations of other specific sugar sequences were found to correlate with p53 expression in certain subtypes of gastric carcinomata. These involved PSA, GNA and NPA in low-grade carcinomata; LCA, VVA and LTA in intestinal type tumours and l-PHA, SNA, LFA, PTA and CTA in poorly-differentiated tumours.

The mechanisms of the relationship between the changes in glycans and p53 expression is not clear. One postulation is that some sugar transferases may maintain their previous or altered activities in the whole process of tumour occurrence and progression under the influence of p53, so that the synthesis of these sugar chains containing those glycan sequences were more abundant, as detected by lectins above, than the others which might be suppressed in the meantime.

An alternative hypothesis is that the overexpressed p53 protein is modified by glycosylation, which leads to the increased staining for certain types of glycans. This proposition is supported by Shaw *et al* (1996). In their recent report, they showed that this p53 was O-glycosylated by *wheat germ* agglutinin (WGA) chromatography and galactosyl-transferase labelling. It was found that at least one of the sugar residues masked the epitope specific for a monoclonal antibody, PAb421, which recognised a domain of the carboxy-terminus of p53

contains a basic region for DNA binding. This masked epitope could be re-exposed and made reactive with PAb421 after digestion with hexosaminidase. This suggests that the addition of sugar groups to p53 protein is involved in the inhibition of the ability of p53 protein to bind to DNA and could contribute to the alteration in gastric glycans.

However, as discussed above about the Rb protein expression, the glycosylation of p53 protein may not be the main factor for the alteration of glycans, since it is also present in only nuclei and can not explain the massive changes of staining patterns of glycans in the cytoplasm and membranes. It, therefore, supports the proposition that alterations in glycans may result from the changes of glycosyl transferases and they may also be the consequence of cellular reaction to the oncogenic growth and proliferation.

9.5 PCNA STAINING

PCNA is a nuclear antigen which represents the proliferative status of a cell. Tumour cells, at least of the growth fraction, are usually under constant growth and division, however slow, PCNA demonstrated those cells of the growth fraction which were at the appropriate part (Late G1 and S phases) of the cell cycle.

The results showed that proliferation, as indicated by PCNA, became progressively more marked as the tumour cells became more anaplastic and fewer were differentiated. This was reflected in the relatively lower proportion of PCNA-positive cells in well-differentiated carcinomata relative to the poorly-differentiated and diffuse-type tumours. It was also noted that the PCNA-positive cells were often abundant and intensely stained in the invasive margins of tumours, reflecting very active cell division at those sites.

It was evident that some glycans altered in relation to the elevated expression of PCNA. Lectins showing significant difference in staining between PCNA positive and negative tissues include PNA, MAA, SBA and HPA, though some variations were observed between subtypes. Certain lectins showed their preferential staining only in some subtypes of gastric tumour tissues with a high level of PCNA expression. It was also notable that some lectins such as VVA showed diminished binding to those tumour tissues with increased expression of PCNA.

These observations may suggest that certain types of glycan are preferably expressed on cells under active proliferation. Such a glycan might be an enhancer of, or a result of, cell growth.

Glycan changes in neoplasia have been suggested to correlate with the abnormal expressions of both oncogenes and tumour suppressor genes in this study. These changes may be the consequence of the activity of those genes, but they may also be among those factors which affect the status of oncogenes and tumour suppressor genes or their products.

9.6 CONCLUSIONS

1. The expression of two oncogenes (Bcl-2 and *ras*), two tumour suppressor genes (Rb and p53) and proliferative cell nuclear antigen (PCNA) were studied in various gastric tissues. It was found that the abnormal expressions of oncogenes and tumour suppressor genes are common events in gastric carcinogenesis and tumour progression.

2. Bcl-2 protein was, as in chapter 8, found frequently to be expressed in 'precancerous' lesions and advanced gastric carcinomata, with high proportions of positive tumour cells. It was evident that the expression of this oncogene was an early event of tumourigenesis, as

shown in 'pre-neoplastic' lesions and that it persisted in its activity in late tumour progression, as shown in poorly-differentiated carcinomata which had high frequencies of 'positive' cases and proportions of 'positive' tumour cells. The mechanisms of the abnormal expression of this protein was not clear, but it might well have occurred because the oncogene itself was activated, leading to the elevated expression of its oncoprotein. Alternatively, the metabolism of the protein may have been changed and its degradation partially blocked, so leading to an increase in its pool size. A third possibility was that a lack of, or change in, the receptors for Bcl-2 oncoprotein (the nuclear anchor), resulted indirectly in its accumulation. The increased expression of Bcl-2 protein could prolong the cell cycle and increase the risk of activation of other oncogenes and potentiate the occurrence of a malignancy. Therefore, elevated expression of Bcl-2 oncoprotein could contribute to both gastric tumourigenesis and progression.

It was found that the abnormal expression of Bcl-2 protein was associated with alterations of glycans detected by lectins, such as PNA, MAA, SBA and HPA. It was not clear whether Bcl-2 protein had a direct effect on the expression of glycans. This was considered made in chapter 9.

3. The *ras* oncoprotein was abnormally expressed in a high proportions of tumours and a 'abundant' tumour cells were noted. These findings further support the postulation that *ras* protein was involved in the gastric carcinogenesis. Since its elevated level of expression retained in advanced tumours, it is possible that *ras* protein was also involved in tumour progression. It was also noted that elevated expression of *ras* protein was closely related to the altered glycans detected by PNA, MAA and HPA. The mechanisms underlying this might reflect the elevated activities of certain glycosyl transferases, as reported elsewhere (Easton *et*

al, 1991; Wojciechowicz *et al*, 1995), but it is still unclear whether p21 influences the activities of those glycosyl transferases directly or indirectly.

4. The elevated expression of Rb protein was only found in gastric carcinoma tissues and not in 'precursor' lesions. A hypothesis was proposed in which a suppression of Rb gene expression occurred in the 'precancerous' stage. This blockade was released or the gene was mutated during a later stage in carcinogenesis and so resulting in the increased expression of Rb protein. The Rb protein detected could have been a mutated, non-functional product, or it could be normal Rb protein which had become sequestered by other mutated proteins (such as its nuclear anchors). Rb protein expression was found to have some relation to particular alterations in glycans as shown by LCA and UEA-1, and suggestions had been made as to their relation, but whether Rb protein is directly involved in the regulation of gastric glycan assembly remains unknown and doubtful.

5. An elevated expression of p53 protein was frequently seen in gastric carcinomata, which supported the proposition that the altered expression of p53 protein was an early event and was the reflection of mutations of P53 gene. This abnormal expression of p53 protein persisted with tumour progression. It was found that there was a relationship between the abnormal expression of p53 protein and altered glycans, detected by PNA, MAA and HPA. The detailed nature of this relationship was not clear, but it had been noted that the posttranslational modifications of p53 protein were involved in glycosylation. It was, therefore, reasonable to propose that elevated amount of p53 protein might provide an increase in sites for glycosylation, which led to the increase of the glycans which were recognised by lectins.

6. The expression of PCNA was observed in all types of gastric carcinoma, but more tumour cells in low grade carcinomata expressed PCNA than those in high grade ones. Certain types of gastric glycans are more likely to be expressed in cells actively in the cell cycle, as detected by lectins PNA, MAA, SBA and HPA.

7. It was notable that the alteration in glycans detected by PNA, MAA, SBA and HPA showed relations to the expression of Bcl-2, *ras*, and p53 proteins and PCNA. It might imply that these glycans were frequently changed in gastric carcinogenesis and later progression. If this is true, these lectins could be very important markers in studying other mucin-secreting carcinomata.

SUMMARY AND FUTURE PERSPECTIVES

A. Summary of findings

There were several important findings in this study.

1. MAA was different from all the other lectins used, in that it did not show binding to normal gastric mucosal cells, but reacted strongly with most neoplastic cells in most gastric carcinoma tissues. This suggests that the terminal sialic acid with α2,3 linkage to galactose is closely associated with gastric neoplasia.

2. Three lectins, BSA1-B_4, SBA and CTA, showed strong, selective binding to normal surface and pit epithelial cells, while eight others (PSA, LCA, GNA, NPA, UEA-1, LTA, SNA and BSA-II) showed binding to gland cells. A number of lectins stained only mucosal epithelial cells and none of the stromal tissues. These lectins were GNA, NPA, UEA-1, LTA, PNA, BSA1-B_4, MAA, VVA, WFA, SBA, DBA, CTA and BSA-II.

3. The abnormal expression of Rb protein was shown in gastric carcinoma tissues. 25 of 47 cases (53%) showed abnormal expression of Rb proteins in about 30 to 40% of tumour cells. This implies that the overexpression of Rb protein was frequently present during gastric carcinogenesis and tumour progression.

4. The sugar sequences, that bind to the lectins PNA, MAA and HPA, have been found to have some connection with the abnormal expression of oncogene proteins and tumour suppressor gene proteins.

B. Further comments and proposals for future studies

1. MAA was clearly distinct from the other two sialic acid binding lectins, SNA and LFA, in that it showed strong binding to most tumour types and cells. On the basis of the known binding requirements of these lectins, this result means that the synthesis of glycans bearing terminal sialic acid with α2,3 linkage to galactose appeared to be enhanced during gastric carcinogenesis and tumour progression. From the literature (Knibbs *et al*, 1991), it is known that MAA has an absolute requirement for a terminal N-acetylneuraminyl residue, specifically with α2,3 linkage, and the subterminal residue must have a β-galactose configuration. It is not clear whether MAA could bind to sialyl residues α2,3 linked to β-N-acetyl-galactosamine, but it has never been shown to do so and itdoes not bind to derivatives of α-N-acetylgalactosamine. However, MAA does not show binding to α2,6 linked sialic acid.

Direct sugar inhibition could not be used as a control, since pre-mixing MAA with sialyl α2,3 galactose would vary the ionic strength of the solution and so change two variables at once. Glycosidase digestion, with neuraminidase, greatly diminished the stain with MAA, providing strong supporting evidence for terminal sialic acid. Weak acid hydrolysis could have been used to provide similar, though less specific, supporting evidence. Another approach would be to use a rigorous biochemical analysis on proteolytic digests of tumour tissues and to characterise the MAA-binding glycoproteins. The specific glycopeptides would be adsorbed on MAA immobilised by attachment to beads and the glycopeptides would be eluted by competing oligosaccharides or by change of pH. After purification, glycopeptides could be analysed by mass spectrometry or high pressure liquid chromatography (HPLC) on pellicular ion-exchange resins, in

conjunction with sequential enzymatic and chemical degradation. However, the quantities of polypeptide available for analysis, even from large tumours, would be small.

SNA does not bind to α2,3 linked N-acetylneuraminic acid, but sequesters α2,6 linked sialic acid irrespective of its linkage to galactose or N-acetyl-galactosamine. LFA is a lectin of less well defined specificity which binds to sialic acid termini. It can bind to both N-acetyl- and N-glycolyl neuraminic acids and, possibly, to sialyl residues in α2,8 linkage to other sialyl sub-termini.

More rigorous proof that the binding of MAA was exclusively to such glycans (that is, those with an α2,3 linked sialic acid terminus attached to subterminal β-galactose) requires further biochemical analysis.

Thus, evidence adduced in this study strongly suggested that MAA could only bind to α2,3 sialyl terminals linked to galactose.

Several possible mechanisms for the increased expression of the MAA-binding sugar sequence can be proposed, assuming that its structure is sialyl α2,3 galactosyl β1-. First, the activity of α2,3 sialyl transferase could be increased by any of several mechanisms. One possibility is that the gene encoding α2,3 sialyl transferase becomes activated during cell transformation and proliferation. Alternately, the degradation of the enzyme might be slowed down. Either mechanism could result in the overall increase in the amount of enzyme. The activity of pre-existing α2,3 sialyl transferase could have been enhanced by activation of a precursor or by an allosteric mechanism. Alternately, the availability of

acceptor substrate (i.e. glycan with a β-galactosyl terminus) or sialyl donor (CMP-sialic acid) might have been limiting in normal stomach and either or both might have been up-regulated in the neoplasms. It is further possible that all reactants are present in normal gastric epithelia, but are not brought together and so the glycan is not formed, such as occurs in some types of lectin-resistant cell lines. Finally, it is possible that all of the MAA-binding glycan is attached to a single type of peptide core, such as a specific mucin, which is only expressed in the neoplasms. This is unlikely, given the diversity of substrate glycopeptides upon which most glycosyl transferases can act.

There is no evidence that the sialyl terminus is in any way directly responsible for gastric carcinogenesis and later progression. It may reflect altered differentiation, but it can not be part of a simple proliferative response (unlike many other changes in glycans detected in this study), because the increased binding of MAA in tumours has no counterpart in the cell hierarchies of normal gastric mucosa. If this increased binding of MAA in gastric neoplasms is only a secondary phenomenon, it can still be used as an indicator of gastric neoplasia.

It will be important to explore what roles the increased α2,3 sialic acid terminus has in gastric carcinogenesis and tumour progression and at what stage(s) it functions. It might affect the adhesiveness of tumour cells to each other and to basement membrane and so influence metastatic potential. It is also possible that the massive sialylation may have an impact on the response of neoplastic cells to external stimuli, such as cytokines, and on cooperative effects within their membranes associated with signal transduction and membrane fluidity.

Other questions can also be asked. Where are the enzymes for α2,3 sialylation encoded? Is it near any known oncogene, tumour suppressor gene or site of genetic instability? Is there any oncogene or tumour suppressor gene that is regulated by the same transcription factors as the transferase? Does the transferase gene encode any variable sequences or sites of variable splicing and is there any variable site of initiation, as in some other transferases?

These can be further investigated by using a range of techniques of biochemical and cellular analysis. Measurements could be made of the activity and/or the amount of α2,3 sialyl transferase in tumour tissues, in comparison with their normal counterparts, so far as these can be defined. A cellular dynamic analysis can be performed by using cell culture to explore the effects of removing sialic acid or transfecting the transferase gene to determine whether the removal of sialic acid or its increment affects the proliferation of the neoplastic cells, their adhesiveness or response to exogenous signals for apoptosis, as well as their antigenicity. It is also possible to investigate if there is any modification of other genes resulting from the activation of the gene(s) directing the synthesis of sialyl transferase. However, the difficulty of culturing gastric mucosa would make such analyses a major research project.

2. Another important finding was that some sugar sequences, as detected by BSA1-B_4, SBA and CTA, were more likely to appear in the lineage from stem cells to mucin-producing epithelial cells, while other sugar sequences (as shown by PSA, LCA, GNA, NPA, UEA-1, LTA, SNA and BSA-II) were confined to the lineage from stem cells to gland cells. Thus it follows that these lectins could be used in studies of the cell hierarchy underlying the proliferation and turnover of the normal gastric mucosa.

The results suggested that the mucosal epithelial cells, mucin-producing epithelial cells and gland epithelial cells, evolve very differently, as they differentiate from the stem cells, in terms of their synthesis of specific glycans, even though they still have many other features, including some glycans, in common. They all conserved the sugar binding sequences, shown by PNA, WFA, MPA and STA. This implies that only certain glycosyl transferase activities were selectively expressed either in mucin-producing epithelial cells or in gland cells, while other transferases were either not synthesised or remained non-functional, or were expressed at high levels throughout. The mechanisms underlying these variations are unknown, but the range of possibilities would be essentially similar to those considered for α2,3 sialyl transfer above.

By comparing the resemblance of the lectin-binding patterns of tumour tissues with their normal cellular counterparts, it may be possible to throw light on the histogenesis of gastric tumours and to illuminate their underlying biology in terms of the cell hierarchies that they contain.

Since a number of lectins showed selective staining confined to gastric mucosal epithelial cells, but not to stromal components, it may be postulated that some glycosyl transferases were completely absent in the stromal cells or were not activated, or were prevented from functioning by separation from acceptor and/or donor substrates (either through their absolute lack or non-approximation). It should be also noted that there was a general difference of glycans in epithelial cells and in stromal cells, since the O-linked glycans were predominant in epithelial cells while N-linked glycans were strongly expressed in stroma.

Stroma is a very important part of neoplastic tissues. It can affect the growth patterns and progression of a neoplasm. If the epithelial part grows faster that the stromal component, a disorganised, papillary structure will be expected. On the other hand, if the stroma grows faster, it will tend to the development of scirrhous neoplasm.

Those lectins that showed selective binding to gastric mucosal epithelial cells, but not to stromal components, may have an application in diagnosis in aiding the determination of whether a tumour is of epithelial or mesenchymal origin. This may be especially helpful if a tumour is of a diffuse type, but with severe stromal proliferation, in which it can be hard to distinguish epithelium from stromal cells. It is also feasible to use lectins to study other mucin-producing tissues and cells and to develop the studies made here, to analyse events occurring at the epithelial-stromal interface, especially where there is incipient invasion and possible stromal change.

In some areas of a tumour tissue the neoplastic cells may show resemblance to surface cells, while neoplastic cells in other areas look more like gland cells. As mentioned above, the stem cell derivatives can differentiate along either pathway. In the process of differentiation and proliferation, some glycosyl transferases are produced or activated, while others may still remain inactive or are not expressed. However, those 'inactive' enzymes, or their genes, may become active (or expressed) in inappropriate ways during tumourigenesis and progression, so that some sugar sequences typical of the mucin-producing lineage might appear in the non-secreting epithelial cell-like tumour cells, or vice versa. This, in itself, could be a pointer to sites of genetic instability, oncogenes and tumour suppressor genes, if the positions of the transferase genes are known.

3. Another important finding was the abnormal expression of Rb protein in neoplastic gastric tissues. The expression and the roles of Rb protein have not been well studied in gastric carcinogenesis. In this study, it was found that Rb expression was not seen in normal and 'precursor' gastric tissues, but it was observed in a high proportion of tumour tissues and cells.

In normal tissues, Rb protein is hardly detectable. It serves as a cellular 'brake', binding, in its hypophosphorylated form, to DNA and leading to G1 arrest. This inhibits the cell cycle from progression into its synthetic (S) phase if DNA has been damaged. Following G1 arrest the cells with damaged DNA will either undergo repair and then re-enter the cell cycle, or they will undergo the process of programmed cell death (apoptosis), so that further entrenchment of the damage will not occur as a consequence of semiconservative DNA synthesis and, hence, will not be perpetuated in the genome. Thus dysregulated cell growth and the risk of a malignancy will be prevented or diminished.

The activity of Rb protein can be altered in carcinogenesis and progression. A range of factors, such as E1A protein from adenovirus, can bind to Rb protein and inhibit its normal function, resulting in unregulated cell growth and the eventual occurrence of malignant neoplasia under the influence of other oncogenic factors.

Based on the observations in this study, it is evident that the expression of Rb protein was increased greatly in gastric carcinoma tissues in that the 'pool size' of immunocytochemically positive Rb protein increased. It is possible that the increased expression of Rb protein is an early event, already present in intramucosal (i.e. intraepithelial) carcinoma (carcinoma in situ), but, if so, it is probably restricted to a subset of 'pre-neoplastic' cells that are rather infrequent and so hard to detect.

The reasons why elevated expression of Rb protein was not seen in 'precursor' lesions is not clear. The quantity of Rb protein expressed may not have been detectable, although it could have been rising, or alternately, the Rb protein was subject to rapid degradation. It was also possible that Rb expression was suppressed until the onset of early gastric carcinoma, or that Rb protein was sequestered by interaction with another protein. With the progression of the incipient neoplasia, more Rb protein was synthesised and released or its degradation was slowed or stopped, leading to its accumulation. as seen in the tumour tissues.

It is not clear whether the increased Rb protein content is of the normal protein or a mutated variant, nor is it known whether it is hypophosphorylated or hyperphosphorylated, since the anti-Rb antibody used was able to recognise all forms of Rb proteins. However, it is most likely that the increased Rb protein was the result of the activation or mutation of normal Rb gene, since a previous study showed that the loss or deletion of this gene was very rare in tumours (Mondon *et al*, 1996). The significance of the elevated Rb protein cannot be fully assessed until the biochemical basis for its accumulation is better understood.

Further work needs to be performed to clarify the nature of the elevated Rb protein and several questions must be addressed. Is it the normal Rb protein? Does it still function normally? Why does the elevated Rb protein appear to be unable to inhibit tumour cell growth, i.e. what prevents this protein from performing its 'braking' role in the cell cycle? Different specific probes and approaches can be employed for the solution these questions. Anti-Rb antibodies directed against different forms of Rb proteins are becoming available and potentially can be used to determine whether the overexpressed Rb protein is hypo- or hyper-phosphorylated, and is normal or mutated. Protein analysis by way of extracting Rb protein from gastric carcinoma tissues or cultured cells can be used to determine if there is any other protein which interacts with Rb protein and thus suppresses its function. It is also feasible to obtain structural information on extracted Rb-type proteins themselves. This might well lead to the identification of another protein, which regulates the function of Rb protein and is often modified in gastric carcinogenesis.

4. Virtually nothing is known about the functional connection between the alteration of expression of gastric glycans and the expression of oncogenes and tumour suppressor genes in gastric neoplasia. In this study, glycan alterations, detected by large panel of lectins were analysed in relation to the expressions of Bcl-2, ras, Rb and p53 proteins. Lectins that were able to show such overall correlation include PNA, MAA and HPA. They showed increased staining in those tissues with elevated expressions of all oncogene and tumour suppressor gene products.

Again, it is not clear if these alterations of gastric epithelial glycans are simply a reflection of the proliferation of the neoplastic cells or are the consequences of biochemical or genetic regulation more directly related to the overexpression of these oncogenes and tumour suppressor genes and/or their products. As explained above, the increase in MAA binding cannot be a simple reflection of proliferation.

Basically, cellular proliferation will eventually affect biosynthesis of glycans, which is an essential part of cell growth and differentiation. Tumour cells usually display extensive changes in many biochemical processes, relative to the normal, if these are actively sought. It is most likely that the alterations in glycans in tumour tissues were the consequence of the overgrowth and abnormal differentiation of tumour cells and that most of them were of a similar nature to other metabolic changes associated with neoplasia.

The possibility can not be excluded that the genes encoding some glycosyl transferases were activated either by oncogenic factors directly, or less directly, by the same factors which activated an oncogene.

A quantitative analysis of the sugar residues, as detected by PNA, MAA and HPA, though difficult, could be made to establish the dynamic changes in the amount of these residues. It would provide crucial information as to what stages in the neoplastic process correspond to the onset of specific alterations in glycans and whether these changes coincide with the expression of specific oncogenes and tumour suppressor genes.

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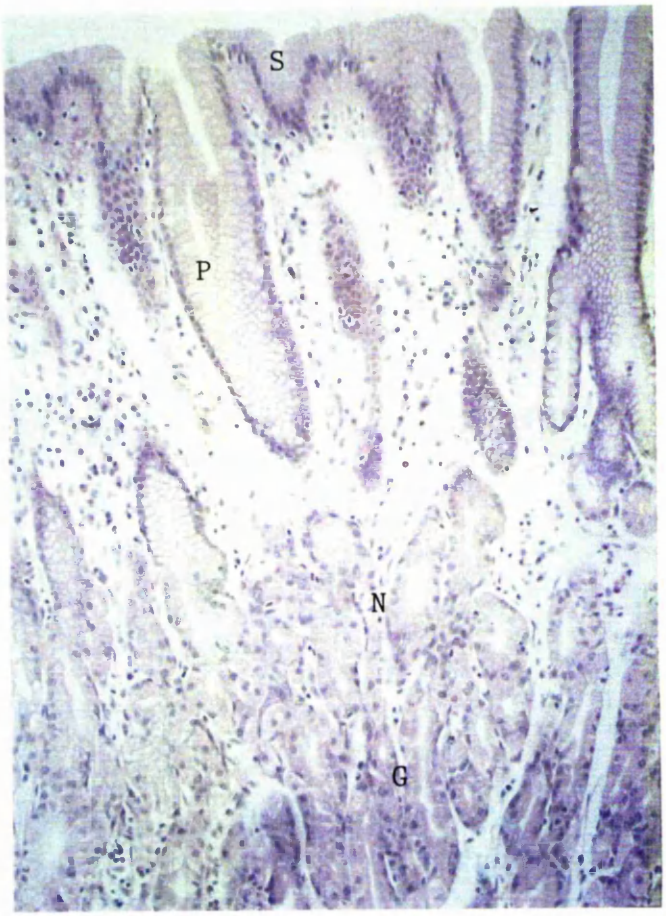

Figure 1.
Normal gastric body mucosa. S: Surface epithelial cells; P: Pit cells; N: neck cells C: Chief cells, G: Gland cells 400X

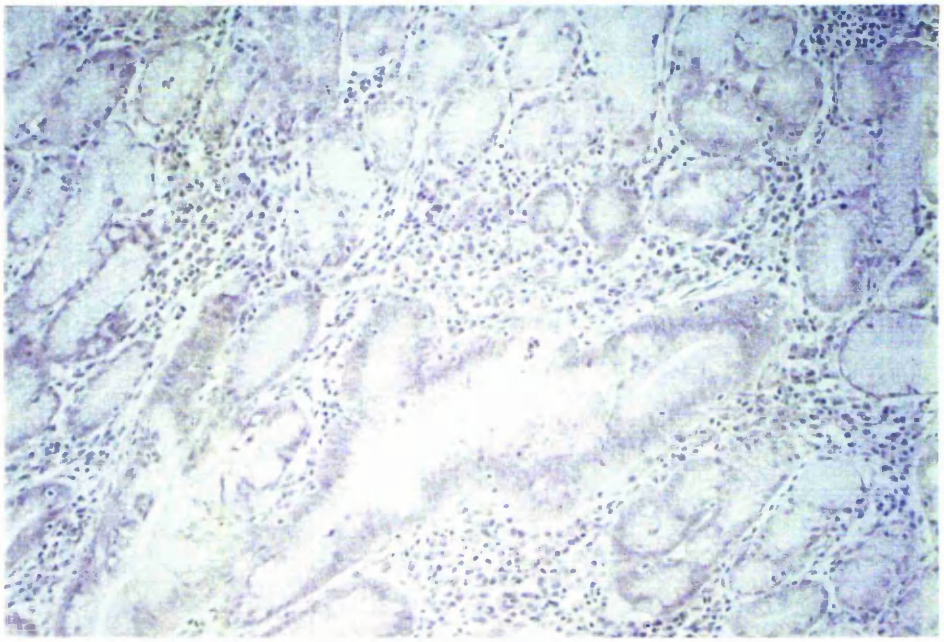

Figure 2. Gastritis and dysplasia. A number of chronic inflammation cells infiltrated in the mucosal layer. Part of the glands were dilated and lined with multiple layers of cells with atypia. 400X

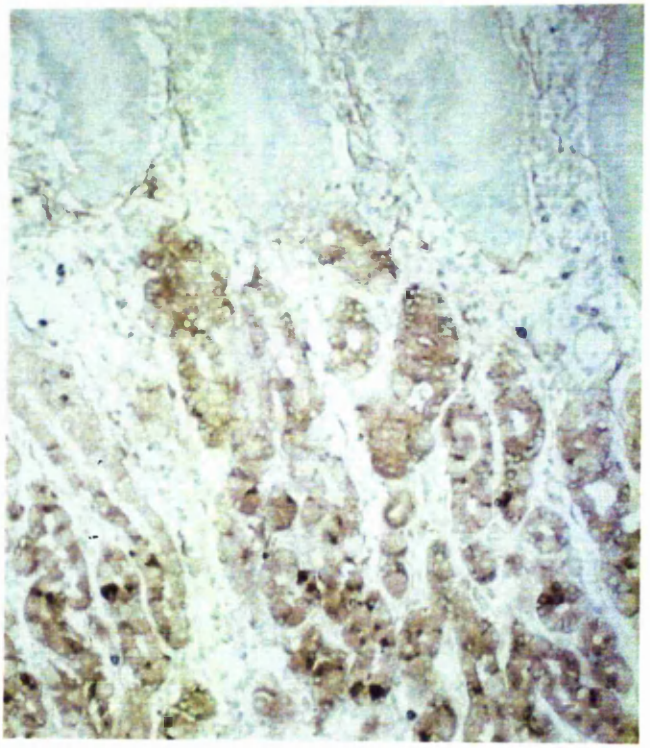

Figure 3.
PSA staining on normal gastric mucosa. Neck and gland cells showed membrane and perinuclear staining. 400X

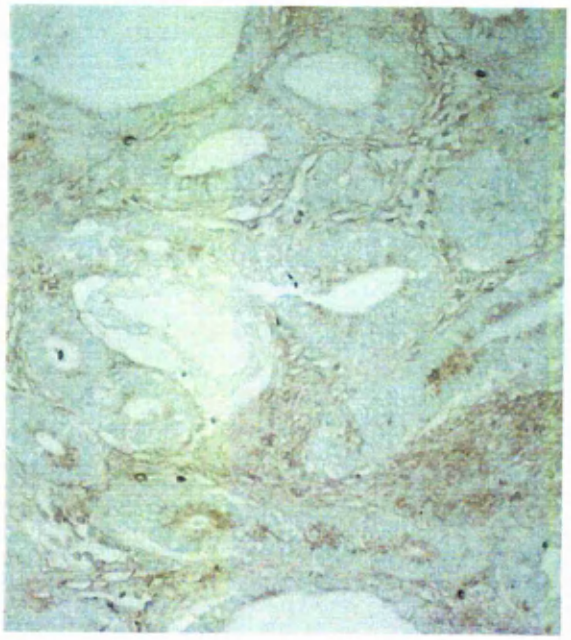

Figure 4. PSA staining on well-differentiated carcinoma. Staining was seen on the lumenal membrane and supranuclear regions 400X

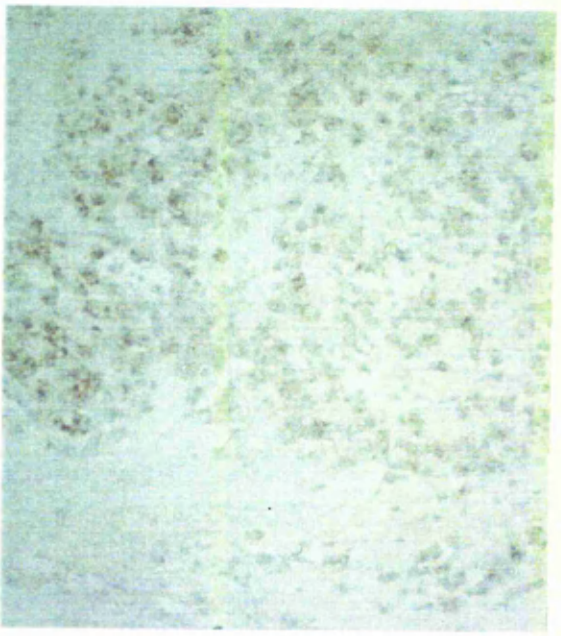

Figure 5. PSA on mucinous carcinoma. Positive cells showed perinuclear staining. 160X

Figure 6. LCA staining on normal mucosal tissues. Positive staining was mainly seen in perinuclear region. 400X

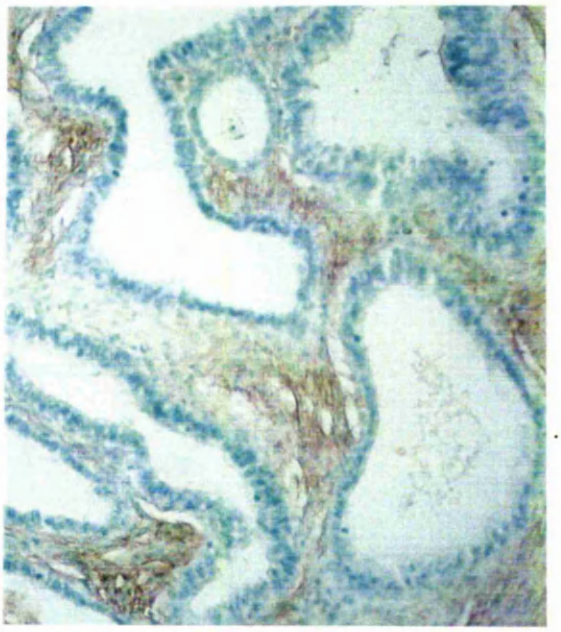

Figure 7. LCA staining on well-differentiated tumour. No positivity was seen on tumour cell surface. 400X

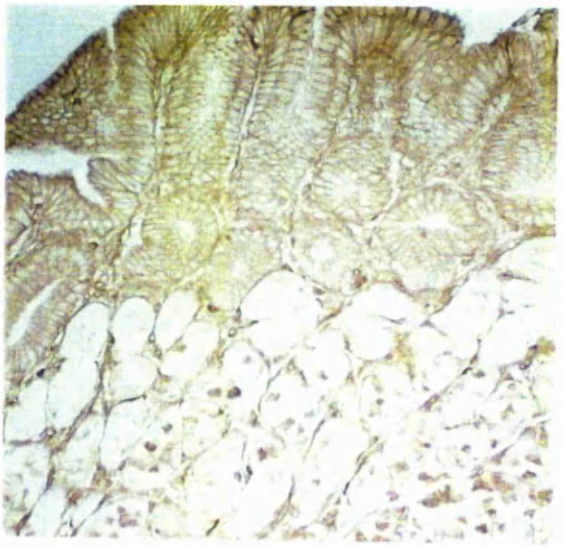

Figure 8. E-PHA staining on normal gastric tissue. The surface and pit cells were intensely stained intercellularly, while neck and gland cells showed nuclear staining. 400X

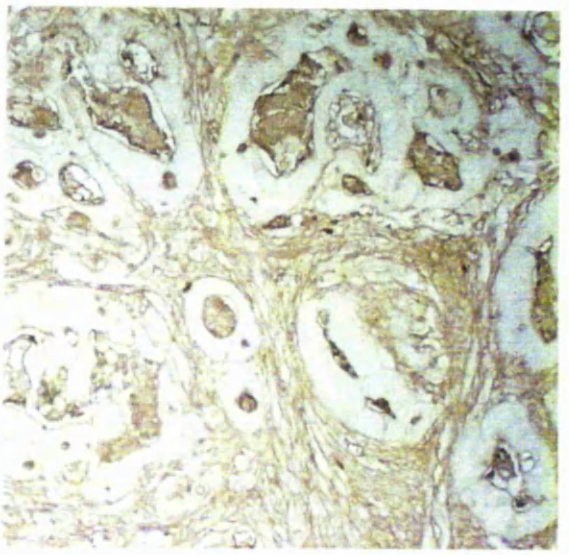

Figure 9. E-PHA staining on moderately-differentiated tumour tissue. Tumour cells showed membrane staining. The secretion was intensely stained. 400X

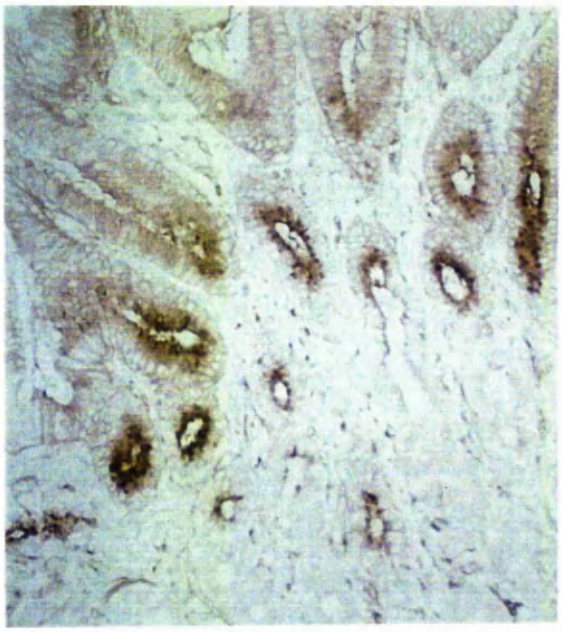

Figure 10. L-PHA on normal gastric mucosa. The epithelium was staining on membrane, while the neck cells were strongly stained in supernuclear regions. 400X

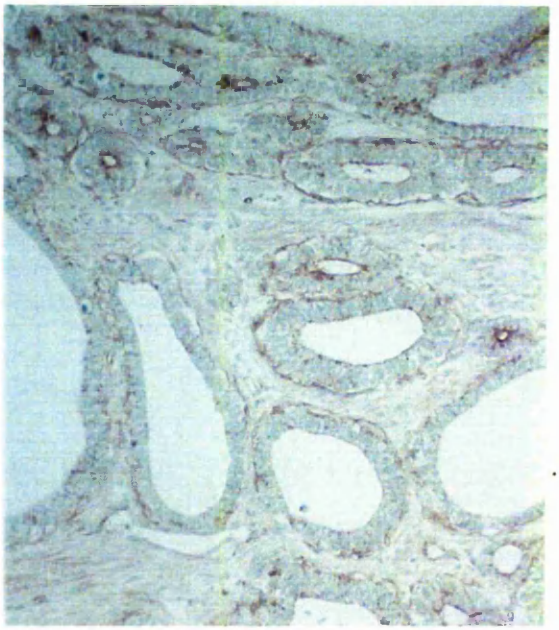

Figure 11. L-PHA on well-differentiated tumour. Staining was mainly on tumour cell lumenal membrane. 400X

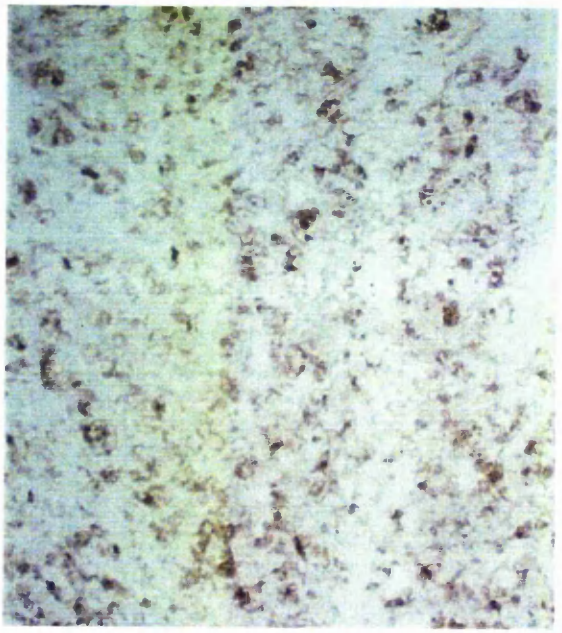

Figure 12. L-PHA on signet ring cell tumour. The tumour cells showed an intense perinuclear staining. 400X

Figure 13. GNA on normal gastric tissue. Strong cytoplasmic staining was seen in neck and chief cells and most parietal cells were negative. 165X

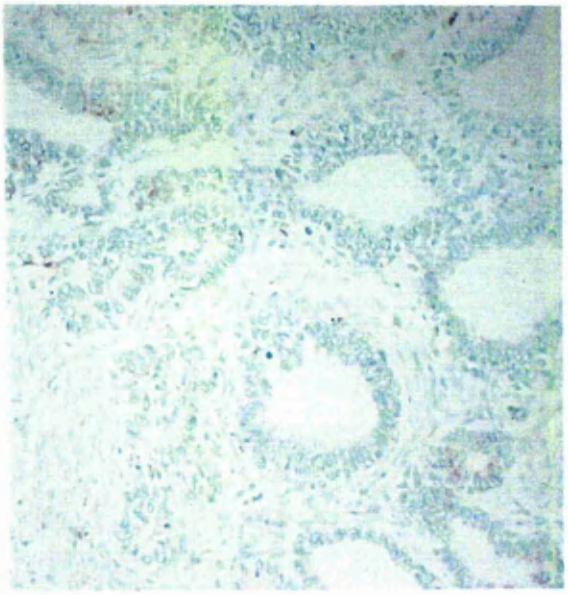

Figure 14. GNA on well-differentiated tumour. Very few cells were stained in nuclear regions. 400X

Figure 15. NPA on normal tissue. Positive staining was seen in neck and glands and the most parietal cells were not shown. 165X

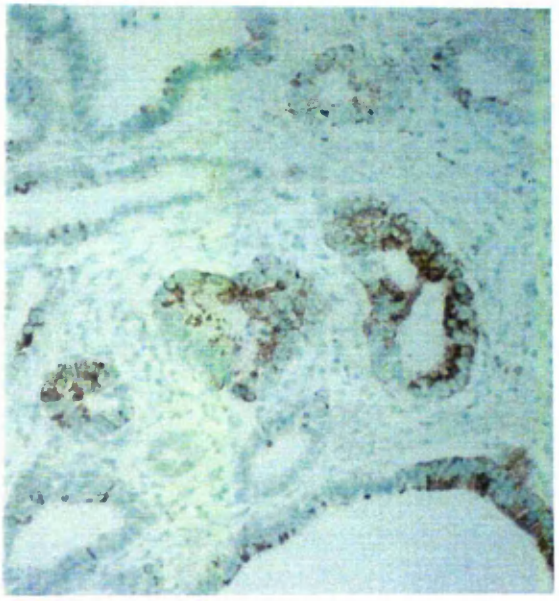

Figure 16. NPA on well-differentiated tumour. Intense staining was seen in perinuclear lumenal cytoplasm. 400X

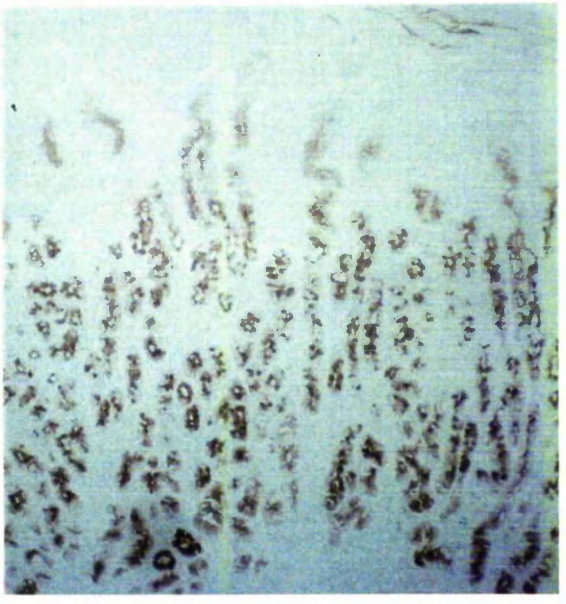

Figure 17. UEA-1 on normal tissue. A few pit cells were positive. Strong staining was seen in neck and gland cells. 165X

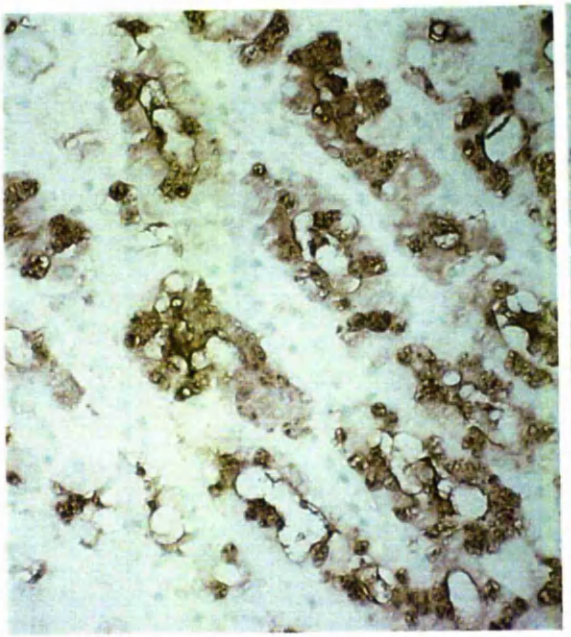

Figure 18. UEA-1 on normal tissue. Chief cells were intensely stained and parietal cells faintly stained. 800X

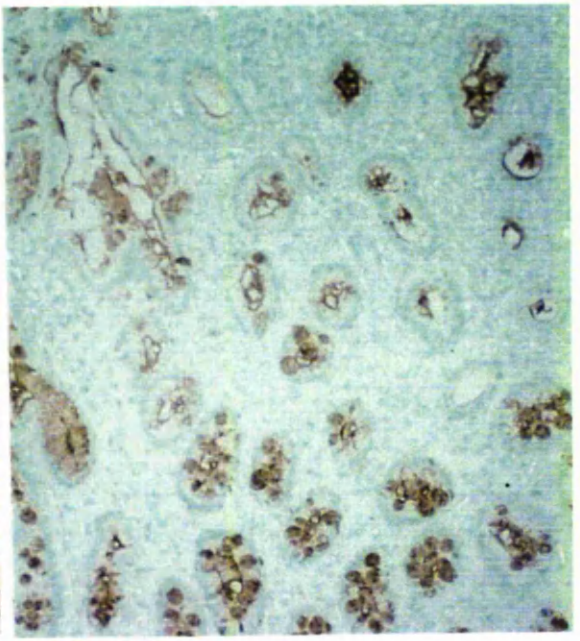

Figure 19. UEA-1 on gastritis tissue. Affected epithelia showed membrane staining. The mucins in goblet cells were intensely stained. 400X

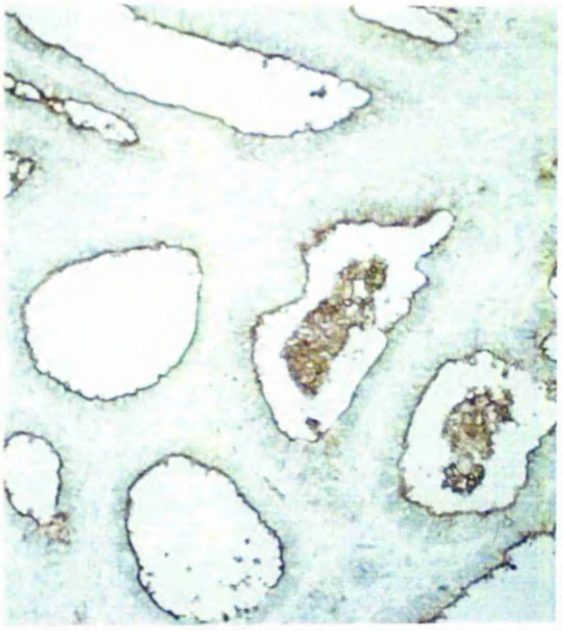

Figure 20. UEA-1 on well-differentiated tumour. Lumenal membrane staining was observed. 400X

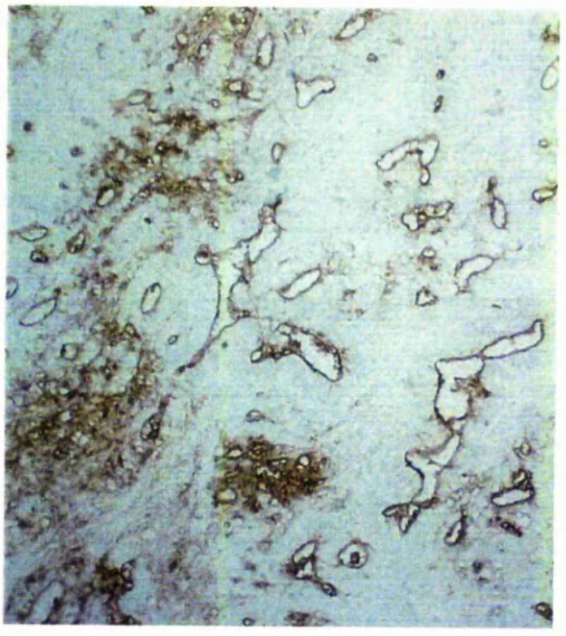

Figure 21. UEA-1 on poorly-differentiated tumour. Positive staining was seen on lumenal membrane. 165X

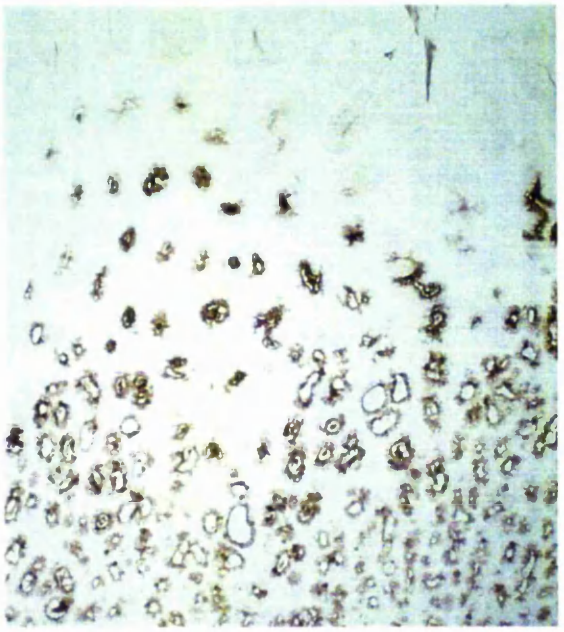

Figure 22. LTA on normal gastric tissue. Staining was mainly seen in neck and gland cells. Stroma was negative. 165X

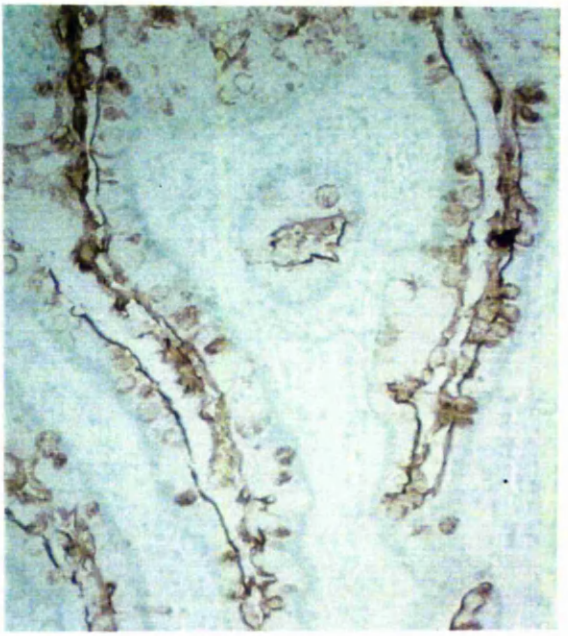

Figure 23. LTA on metaplastic tissue. Goblet cells were outlined clearly. 800X

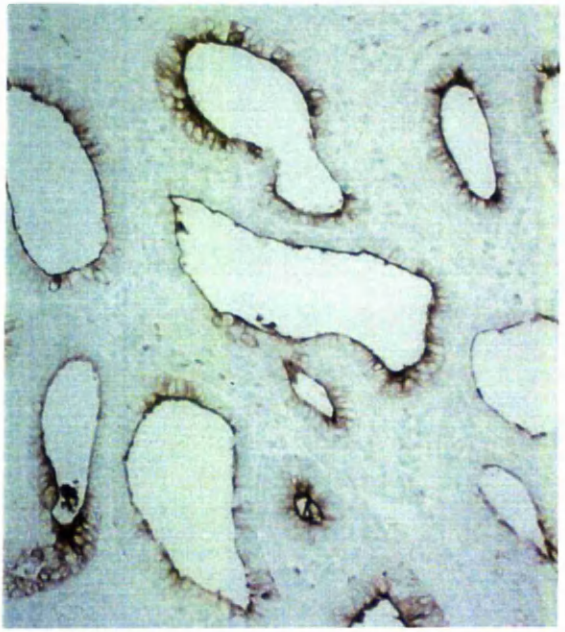

Figure 24. LTA on well-differentiated tumour. Strong lumenal membrane as well as intercellular staining was seen. 400X

Figure 25. PNA on normal gastric tissue. All mucosal epithelium were strongly stained, except for parietal cells. 165X

Figure 26. PNA on normal tissues. Showing strong cytoplasmic staining in chief cells, while weak or negative staining in parietal cells. 800X

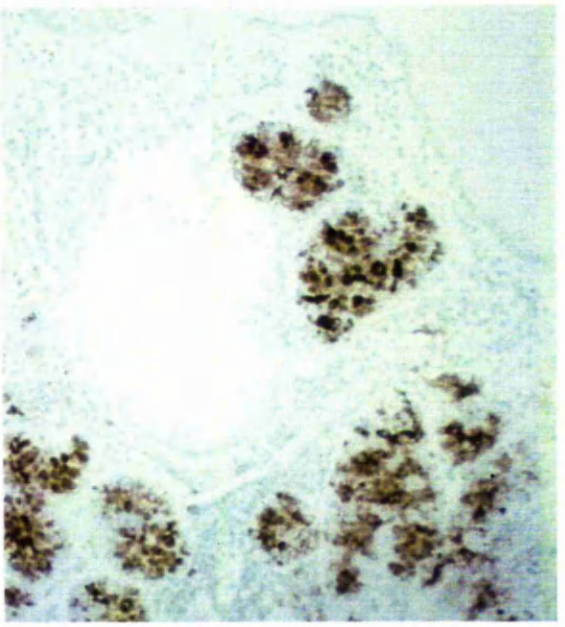

Figure 27. PNA on dysplastic tissue. Intense staining was shown in dysplastic cells.400X

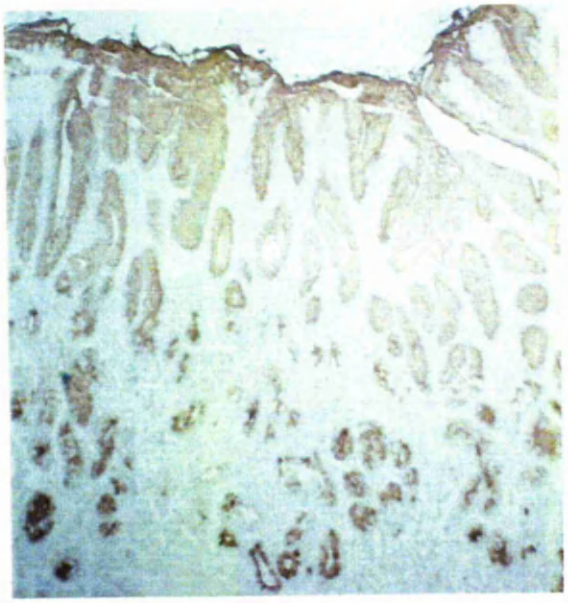

Figure 28. ECA on normal tissue. Positive staining in all cells. 165X

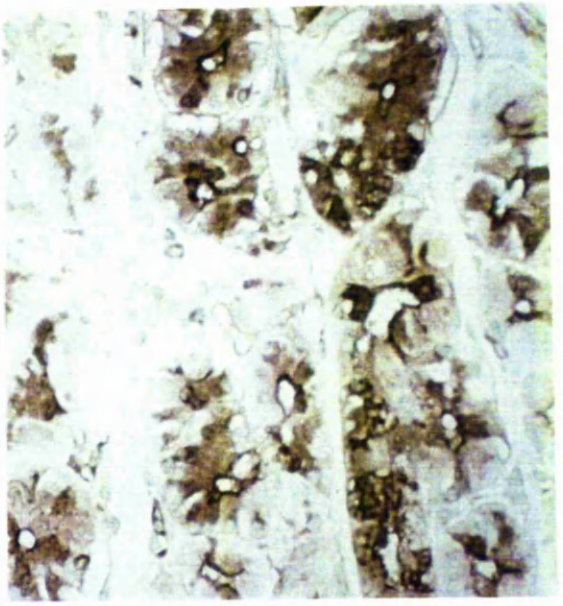

Figure 29. ECA on normal tissue. Chief cells were intensely stained while parietal cells were weak or negative. 800X

Figure 30. ECA on intramucosal tumour. Strong membrane staining was seen in all tumour cells. 165X

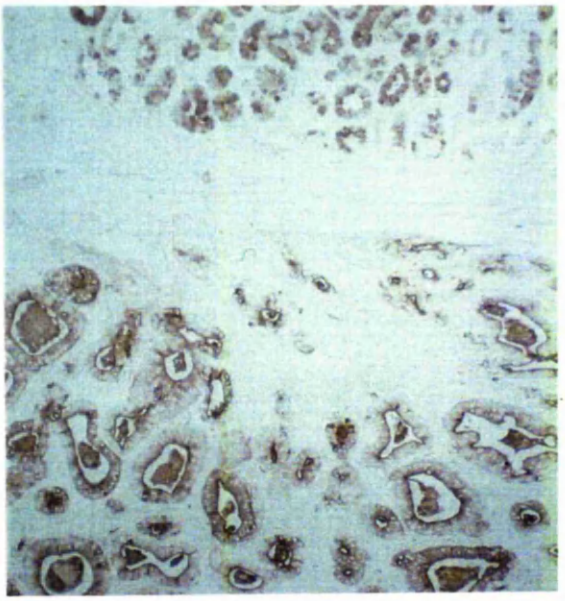

Figure 31. ECA on adjacent tissue. Tumour cells showed intense intercellular and lumenal staining. 165X

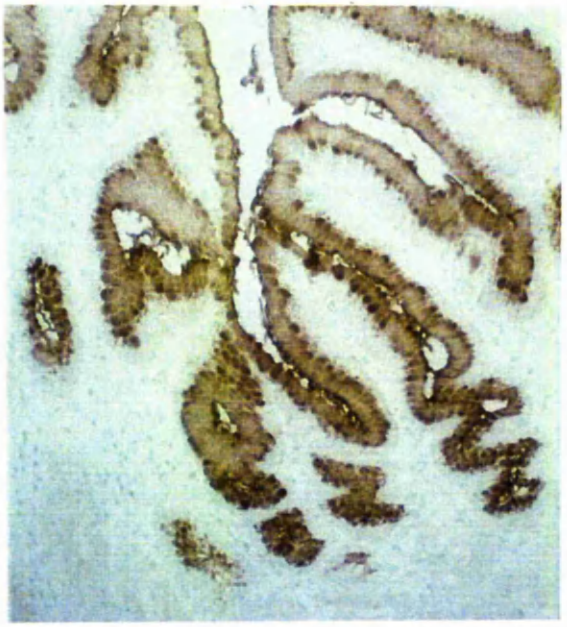

Figure 32. BSA1-B_4 on normal tissue. Positive staining was only seen in mucinous epithelium. 400X

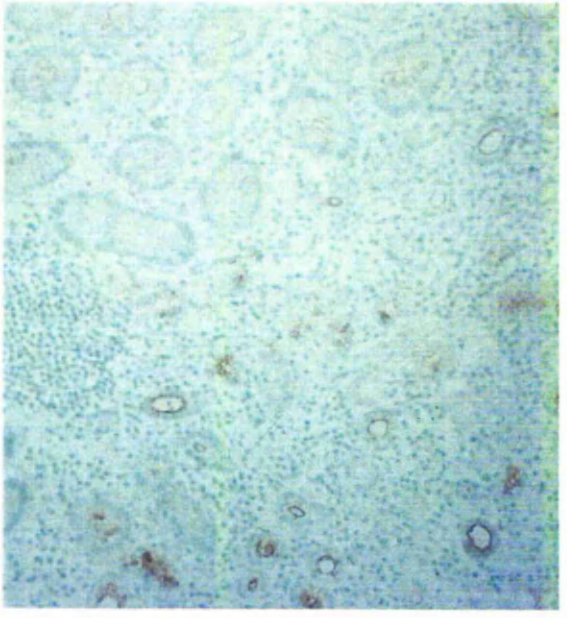

Figure 33. BSA1-B_4 on gastritis tissue. Staining was diminished in epithelium. 165X

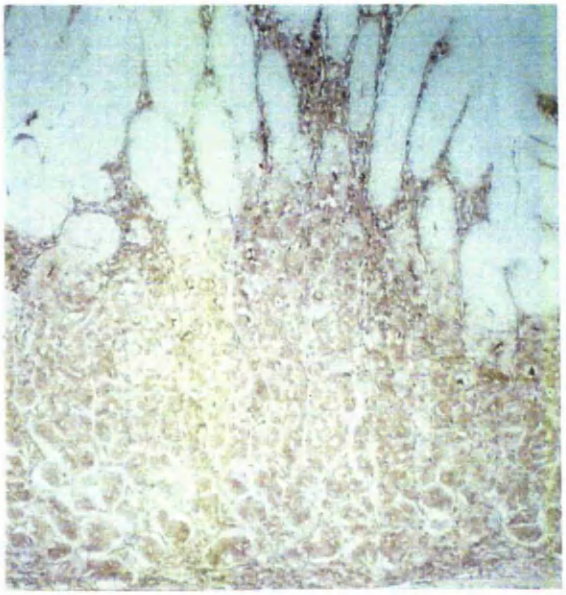

Figure 34. SNA on normal tissue. Staining was seen in the neck and gland cells as well as in stroma. 165X

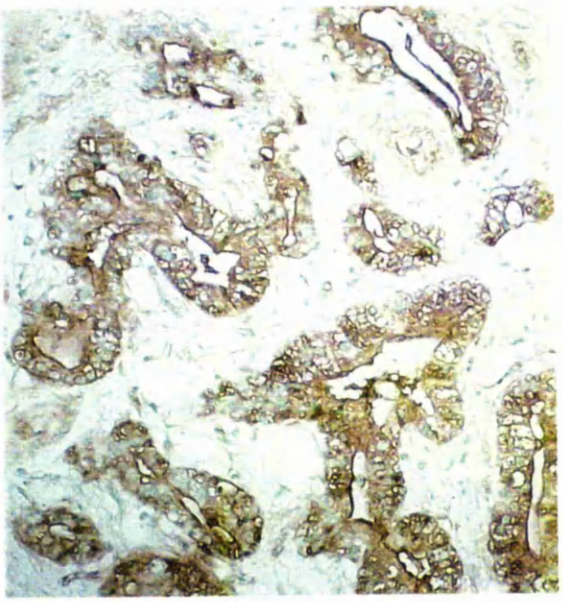

Figure 35. SNA on moderately-differentiated tumour. Cytoplasmic staining was seen in most cells. 400X

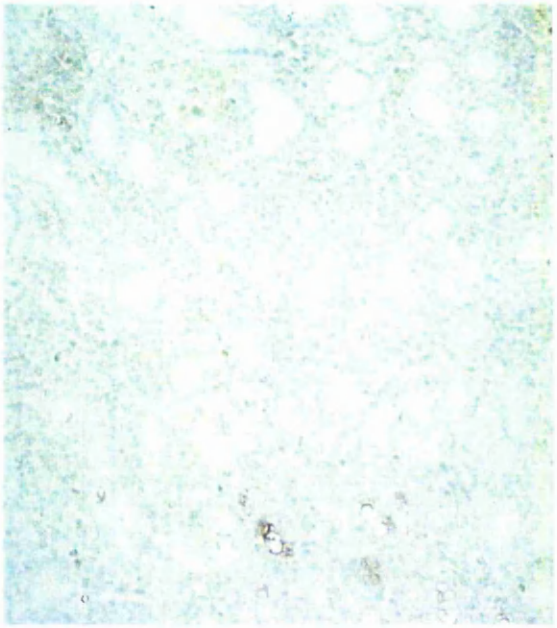

Figure 36. MAA on normal mucosa. Nearly no positive staining was observed. 165X

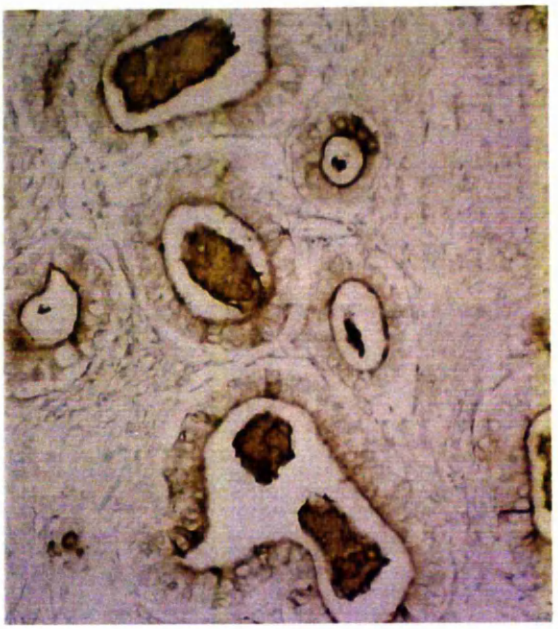

Figure 37. MAA on well-differentiated tumour. Lumenal and intercellular staining was seen. 400X

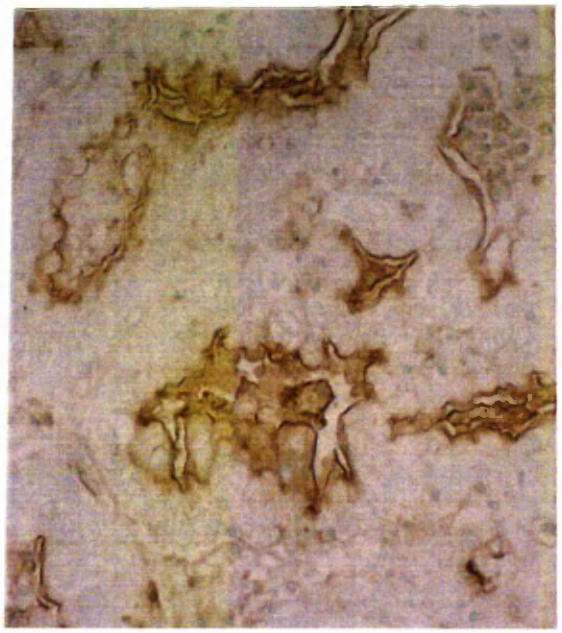

Figure 38. MAA on poorly-differentiated tumour. Lumenal membrane was strongly stained. 400X

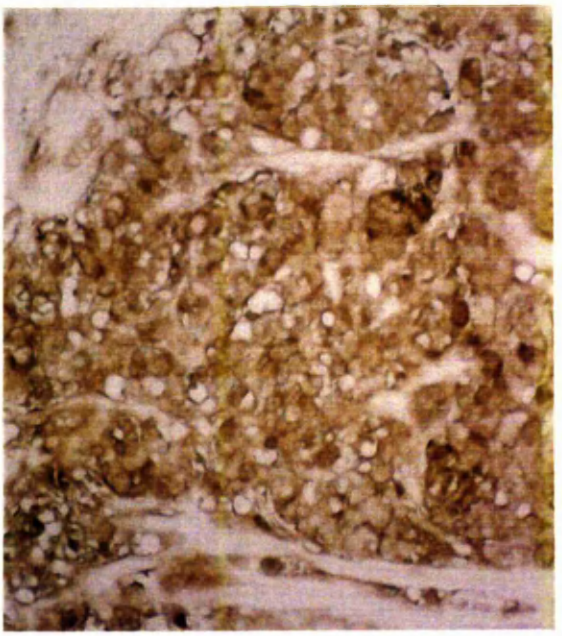

Figure 39. MAA on signet ring cell tumour. Intense cytoplasmic staining was seen in all tumour cells. 400X

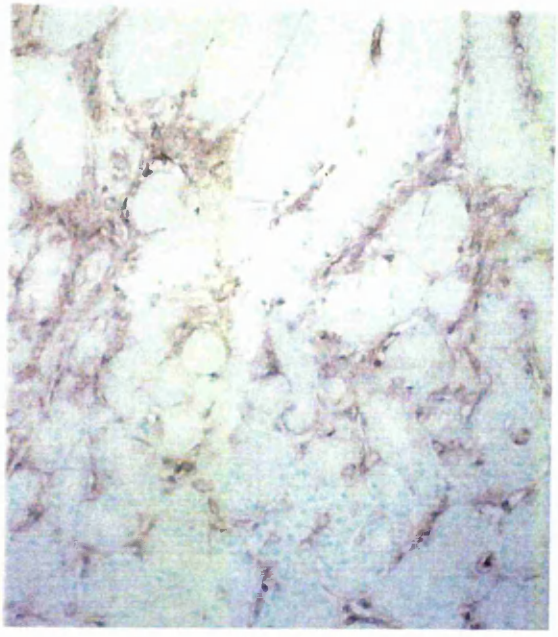

Figure 40. LFA on normal tissue. Mucosal cells showed no positive staining. 400X

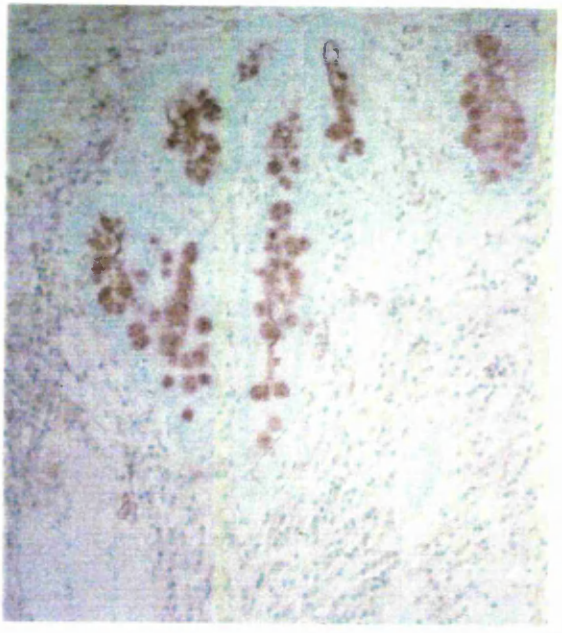

Figure 41. LFA on metaplastic tissue. Goblet cells were clearly shown. 400X

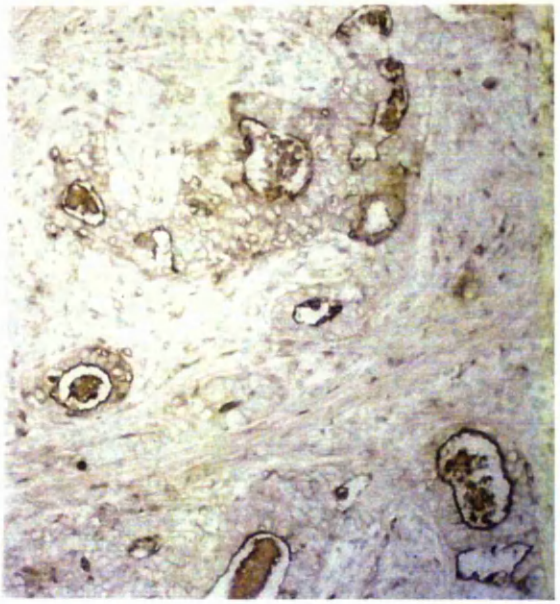

Figure 42. LFA on moderately-differentiated tumour. Staining was seen mainly on lumenal surface and intercellular membrane. 400X

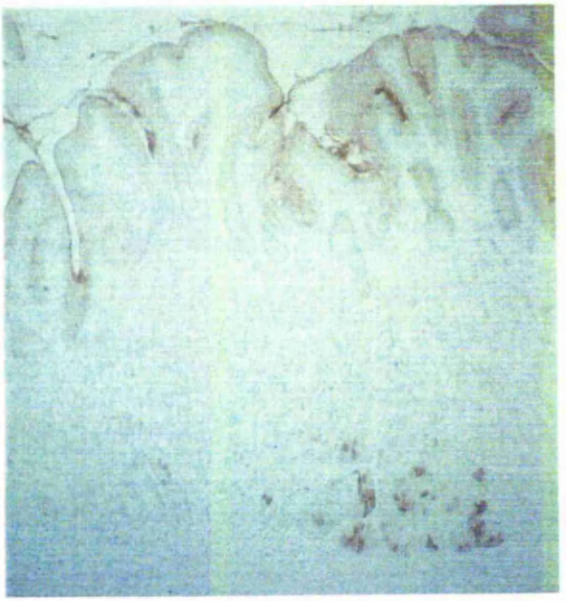

Figure 43. VVA on normal tissue. Mucinous epithelium and few gland cells were positive. Stroma was unstained. 165X

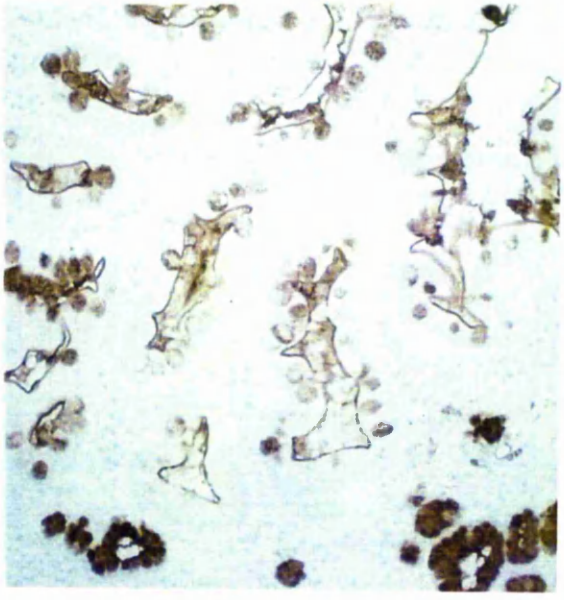

Figure 44. VVA on metaplastic tissues. The Goblet cells were clearly showed by cytoplasmic staining. 400X

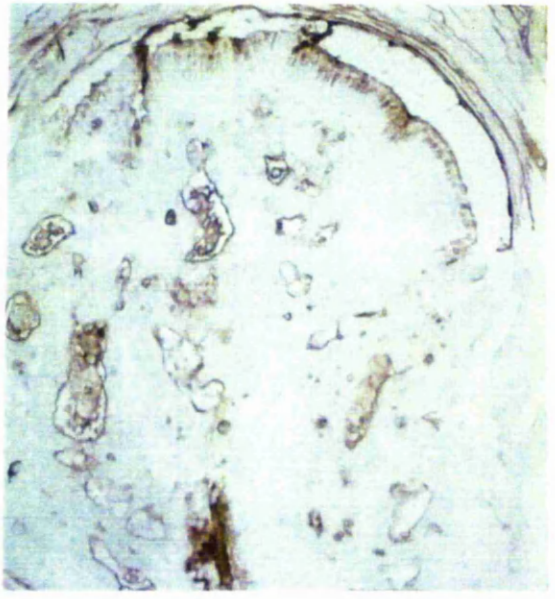

Figure 45. VVA on intramucosal tumour. The lumenal membrane and supranuclear area were intensely stained. 400X

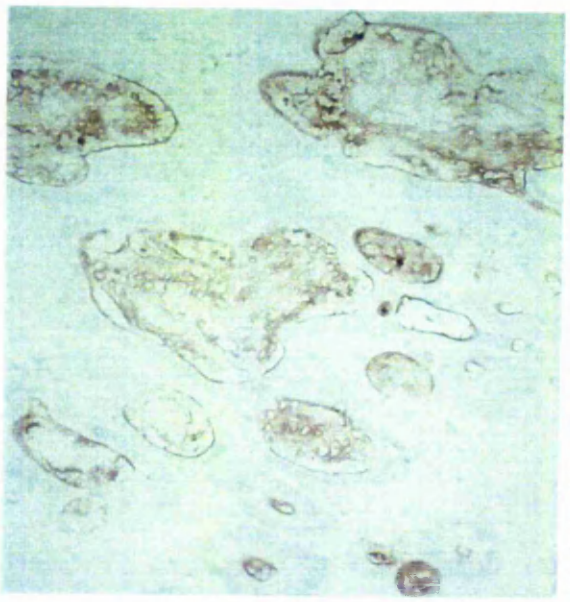

Figure 46. VVA on poorly-differentiated tumour. Positive staining was seen on lumenal membrane. 400X

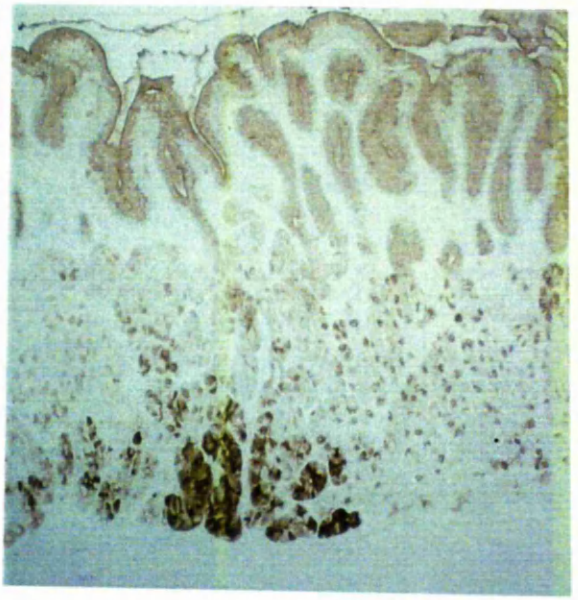

Figure 47. WFA on normal tissue. Mucinous cells were positive in cytoplasm while gland cells were mostly perinuclear staining. Parietal cells were not stained. 165 X

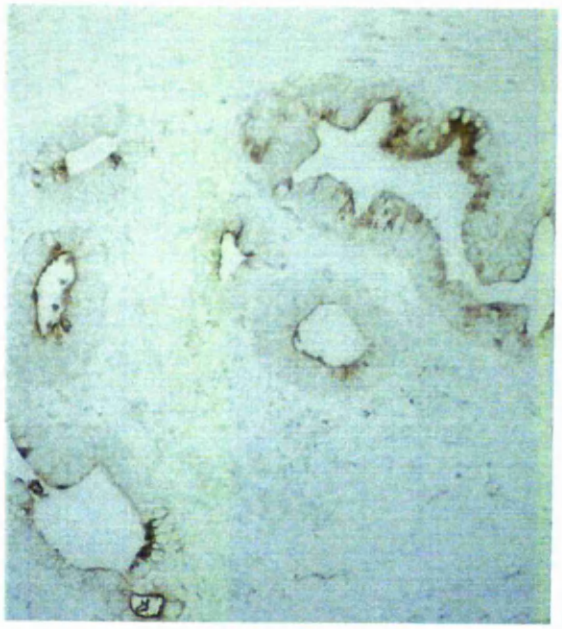

Figure 48. WFA on well-differentiated tumour. Tumour cells showed staining in supranuclear regions and lumenal membrane. 400X

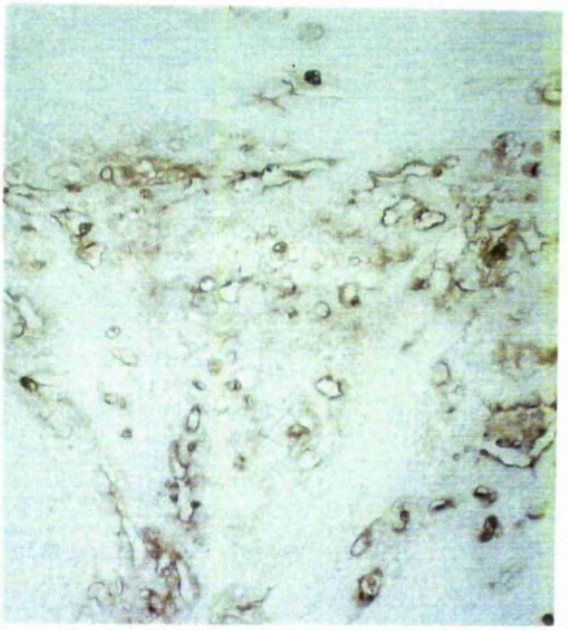

Figure 49. WFA on poorly-differentiated tumour. Tumour cells showed staining in perinuclear regions and lumenal membrane. 400X

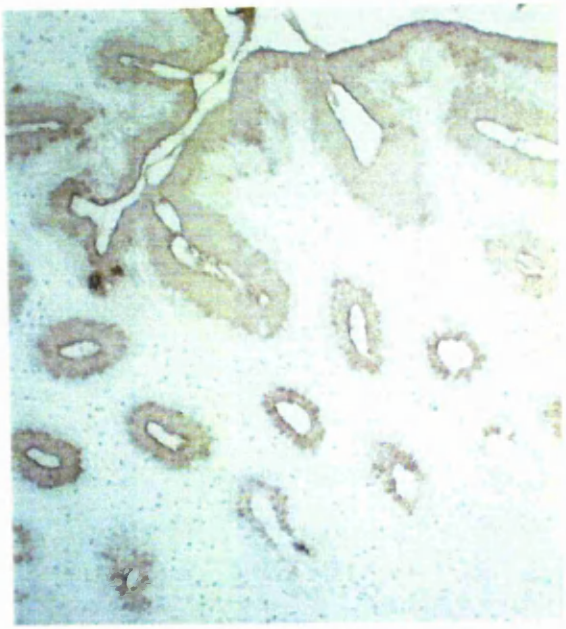

Figure 50. SBA on normal gastric tissue. Only mucinous epithelial cells were cytoplasmically positive. 400X

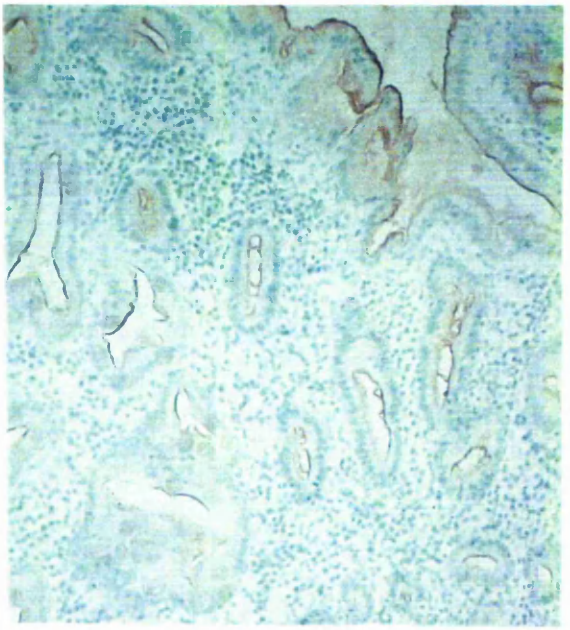

Figure 51. SBA on gastritis tissue. The affected cells showed diminished lumenal membrane staining and some goblet cells were faintly stained. 400X

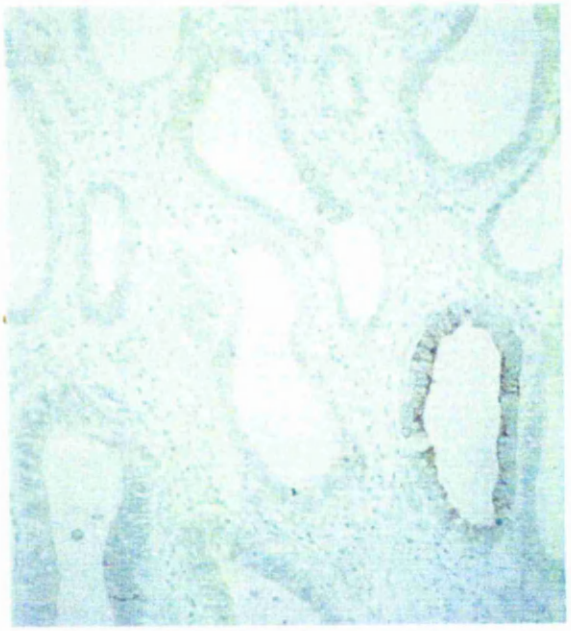

Figure 52. SBA on well-differentiated tumour. Some tumour cells were stained intercellularly and on the surface. 400X

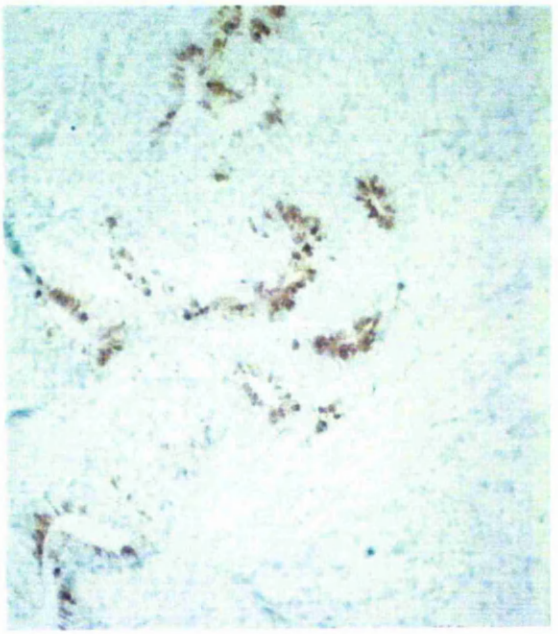

Figure 53. SBA on poorly-differentiated tumour. Part of tumour cells were cytoplasmically positive. 400X

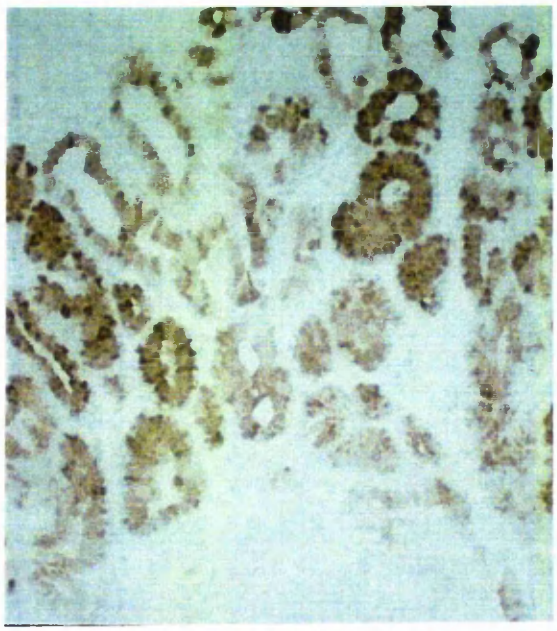

Figure 54. PTA on normal tissue. Only gland cells were strongly stained. There was no difference between chief and parietal cells. 400X

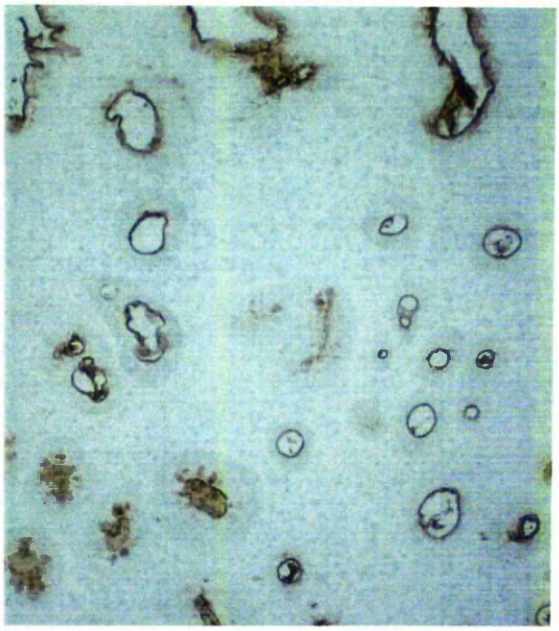

Figure 55. PTA on gastritis tissue. Surface cells showed membrane staining and goblet cells were stained. 400X

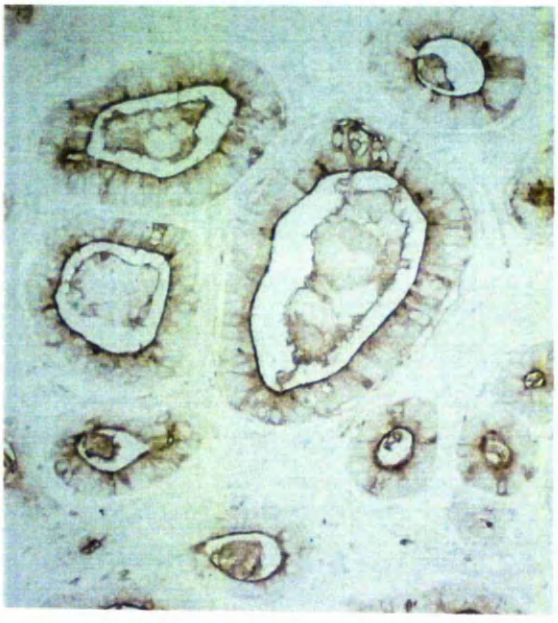

Figure 56. PTA on well-differentiated tissue. Tumour cells showed strong membrane and partial cytoplasmic and perinuclear staining. 400X

Figure 57. PTA on Signet ring cell tumour. Staining was mainly cytoplasmic in most tumour cells. 400X

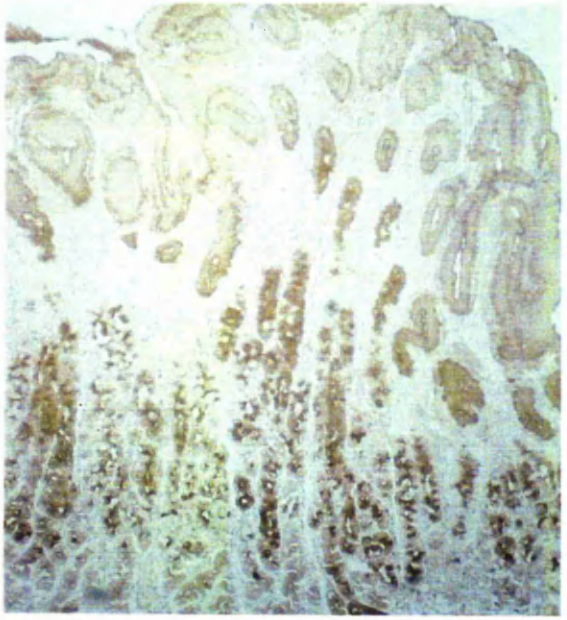

Figure 58. MPA on normal tissue. Overall staining was seen in mucosal cells. 165X

Figure 59. MPA on normal tissue. Differential staining was observed in gland in which chief cells were strongly positive while parietal cells mostly negative. 800X

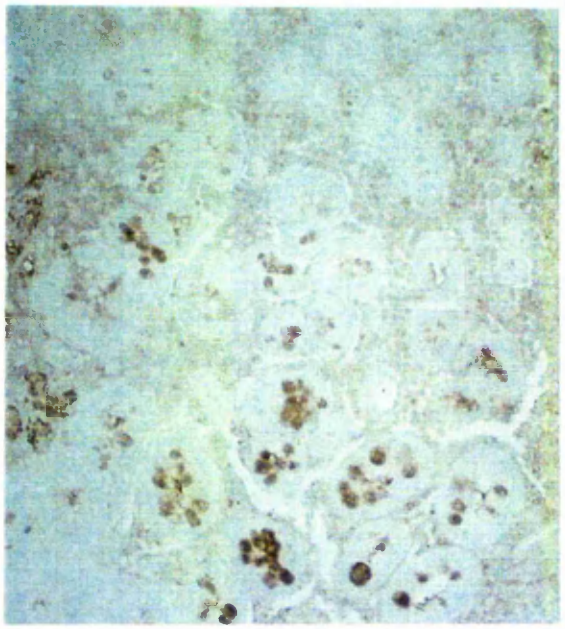

Figure 60. MPA on gastritis tissue. Dminisheded staining was seen in epithelia. Goblet cells were stained clearly. 400X

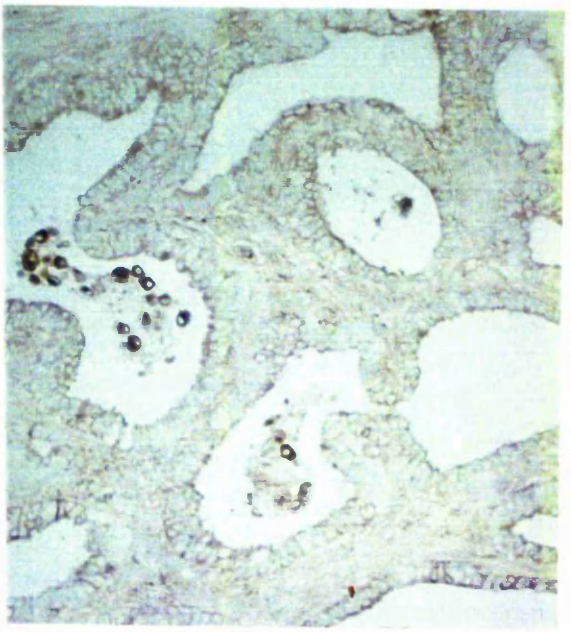

Figure 61. MPA on well-differentiated tissue. Positive staining showed in cell conjunctions and lumenal membrane. 400X

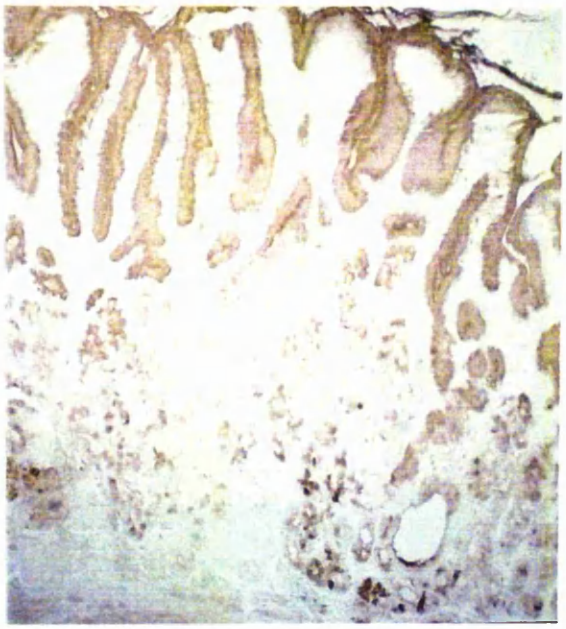

Figure 62. DBA on normal gastric tissue. All epithelial cells and most gland cells were positive. 165X

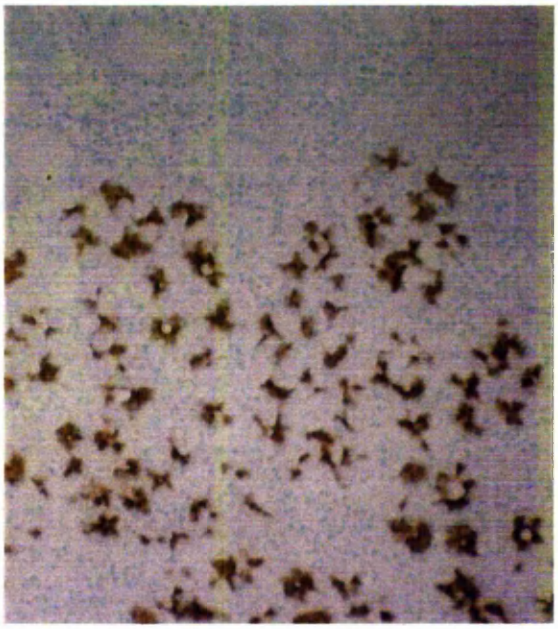

Figure 63. DBA on gastric tissue. Affected epithelial cells lost staining, Chief cells remained staining. 400X

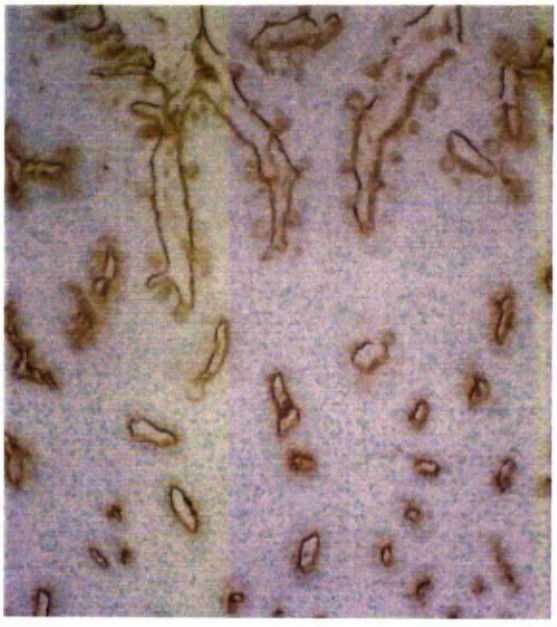

Figure 64. DBA on metaplastic tissue. Epithelial cells only showed membrane staining. Goblet cells were clearly shown. 400X

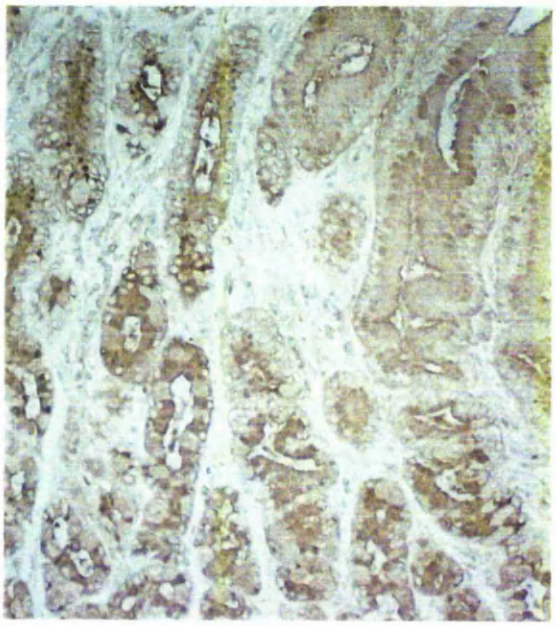

Figure 65. STA on normal tissue. Cytoplasmic staining was seen in most cells except for parietal cells. 400X

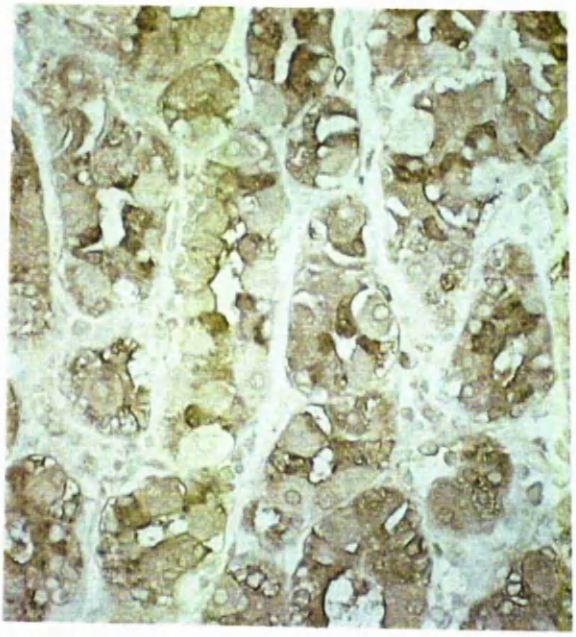

Figure 66. STA on nornal tissue. Chief cells were clearly stained cytoplasmically while parietal cells showed weak staining. 800X

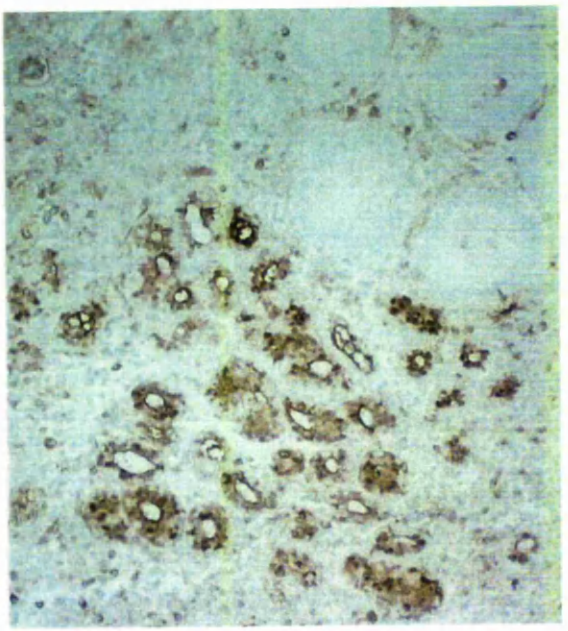

Figure 67. STA on gastritis tissue. Mucinous cells and part of gland cells lost staining. 400X

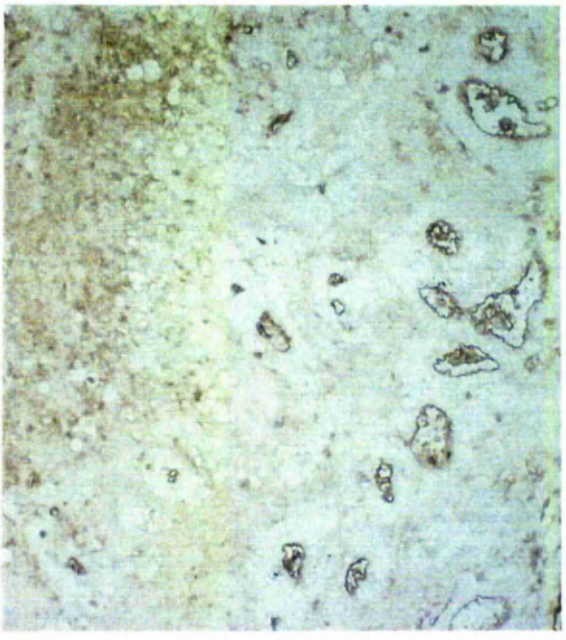

Figure 68. STA on poorly-differentiated tissue. Lumenal membrane and partial cytoplasmic staining were seen in tumour cells. 165X

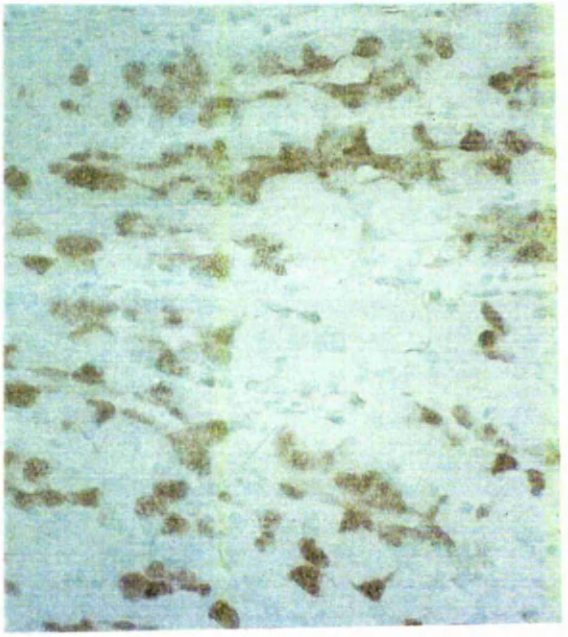

Figure 69. LEA on normal tissue. Showing chief cells with cytoplasmic staining and other cells were unstained. 800X

Figure 70. LEA on poorly-differentiated tissue. Showing membrane staining of tumour cells. 165X

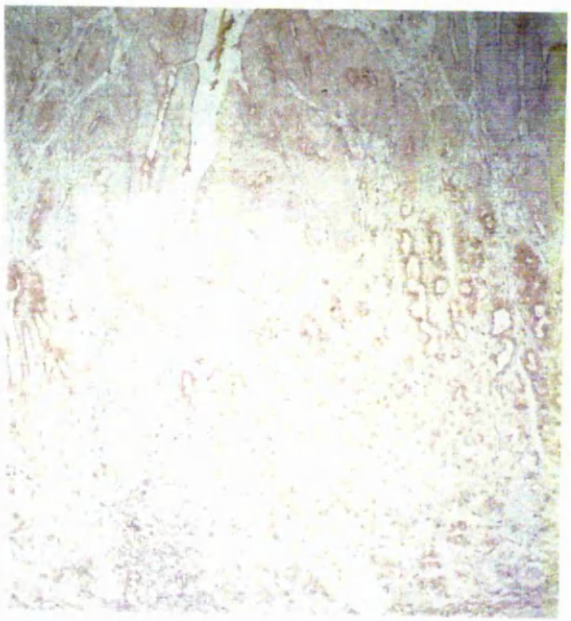

Figure 71. DSA on normal tissue. An overall staining was seen in mucosa. 165X

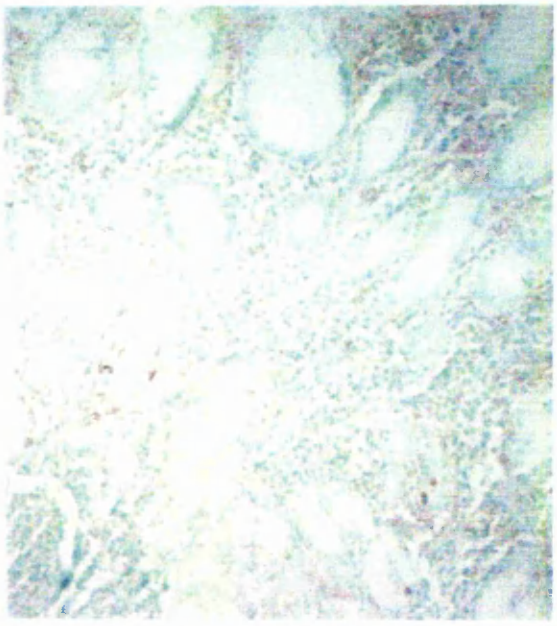

Figure 72. DSA on gastritis tissue. Staining lost in affected cells. 400X

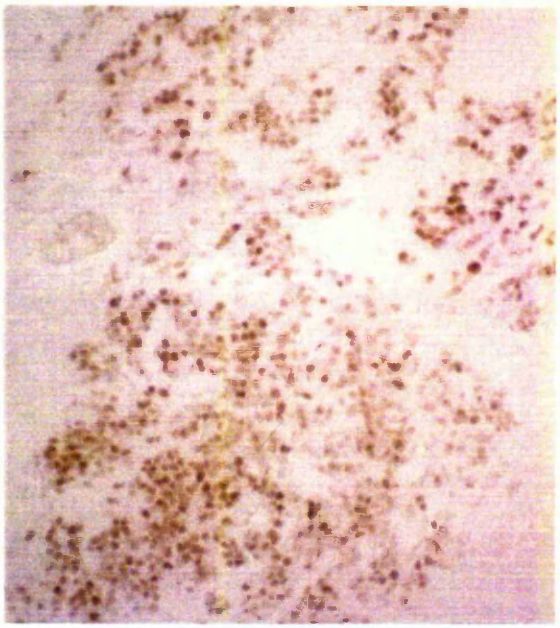

Figure 73. DSA on diffuse-type tumour. Most cells showed scytoplasmic staining. 165X

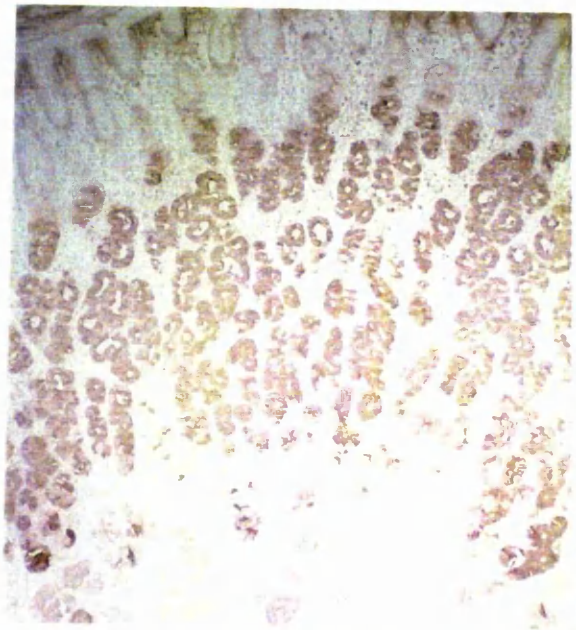

Figure 74. HPA on normal tissue. Surface cells showed perinuclear staining while gland cells were cytoplasmically staining. Most parietal cells were not stained. 165X

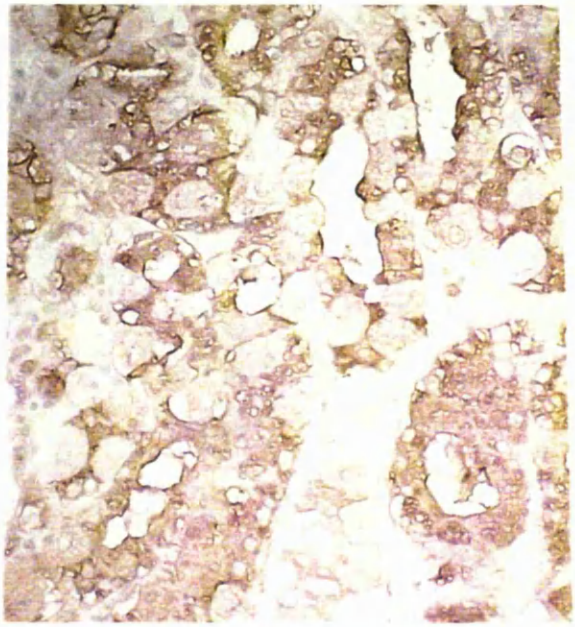

Figure 75. HPA on normal tissue. Showing the contrast of the staining of chief cells with parietal cells. 800X

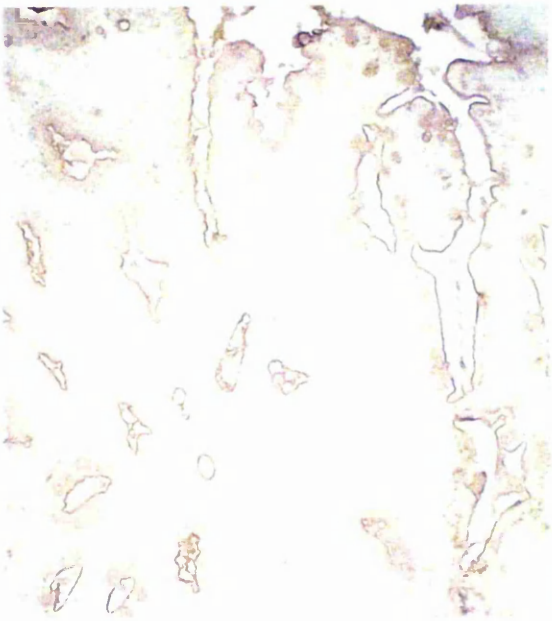

Figure 76. HPA on gastritis tissue. Staining was enhanced in affected epithelial cells and goblet cells were stained in cytoplasm. 400X

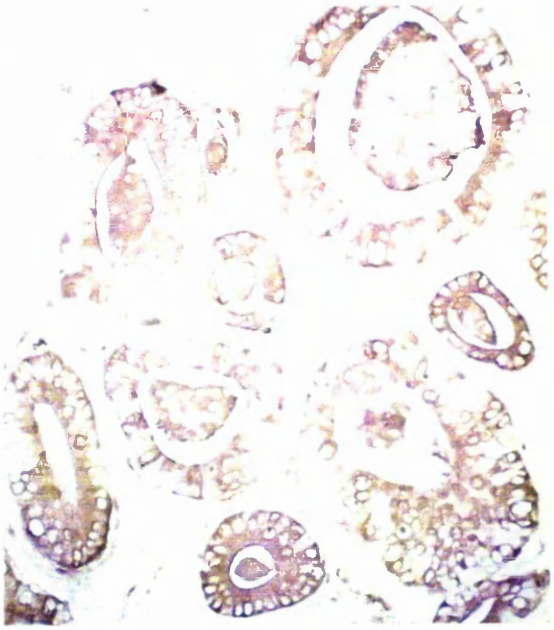

Figure 77. HPA on well-differentiated tumour. All cells showed strong positive cytoplasmic staining. 400X

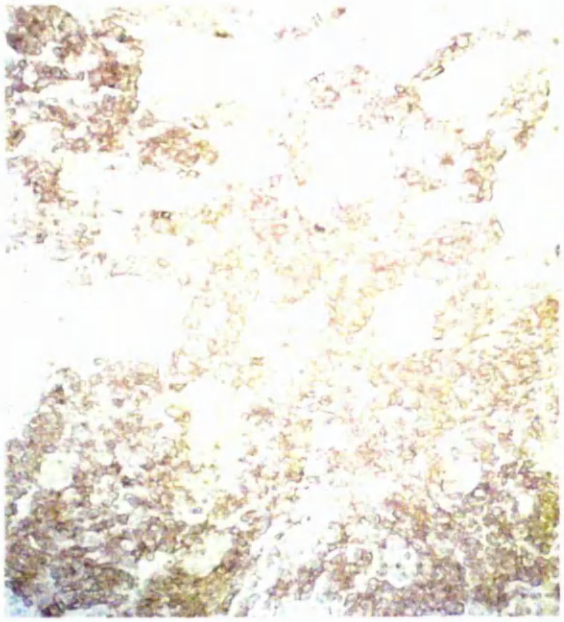

Figure 78. HPA on diffuse type of tumour. Most cells were stained cytoplasmically. 400X

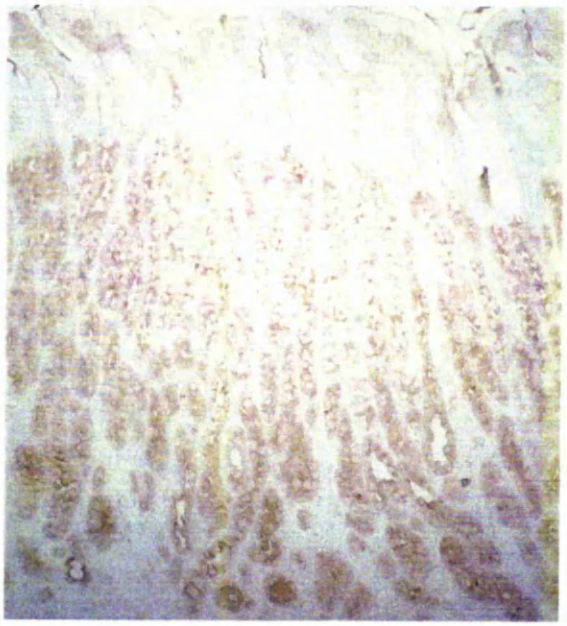

Figure 79. BPA on normal tissue. Gland cells were the main positive cells. 165X

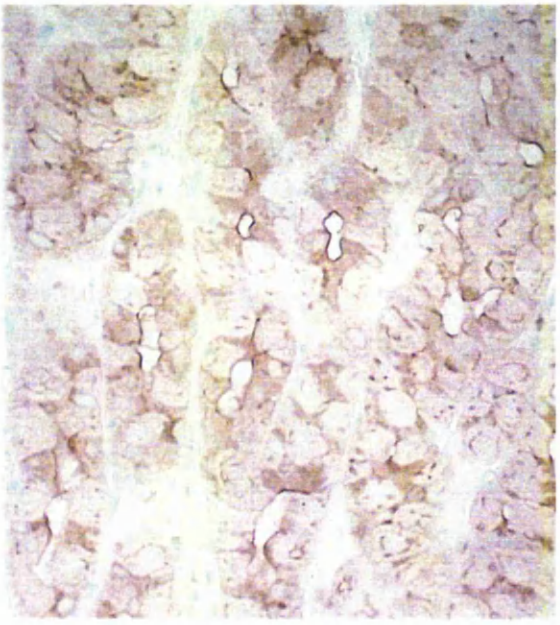

Figure 80. BPA on normal tissue.Chief cells showed membrane and intercellular staining and parietal cells were negative. 800X

Figure 81. BPA on well-differentiated tumour. Few cells showed membrane staining. 400X

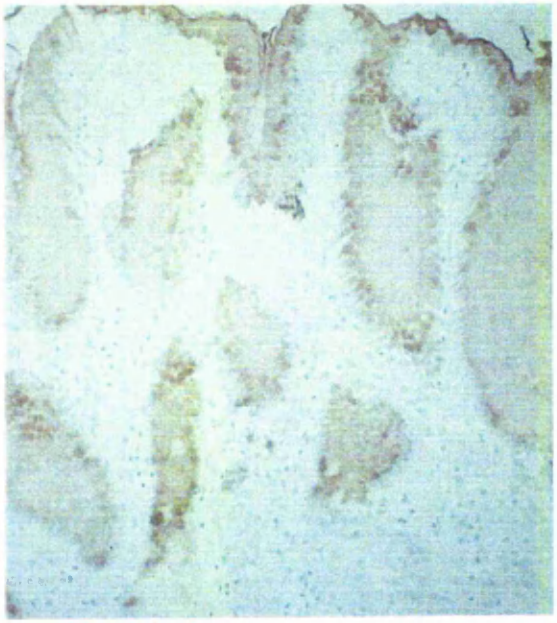

Figure 82. CTA on normal tissue. Only mucinous epithelial cells were positively stained. 400X

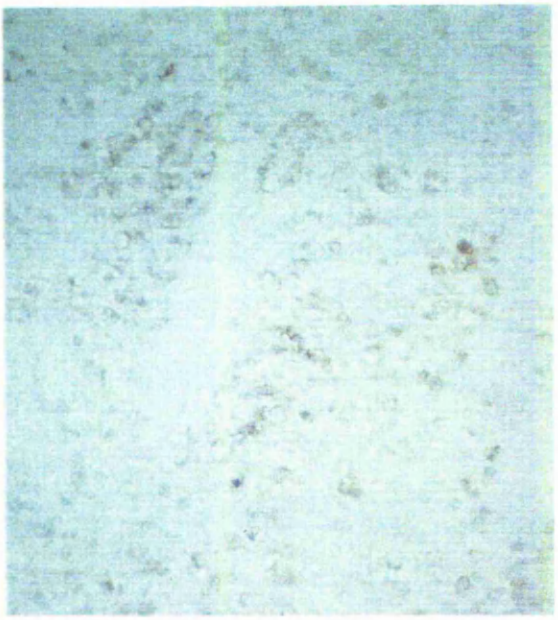

Figure 83. CTA on signet ring cell tumour. Few cells showed cytoplasmic staining. 165X

Figure 84. BSA-II on normal tissue. Only gland cells were intensely stained. 165X

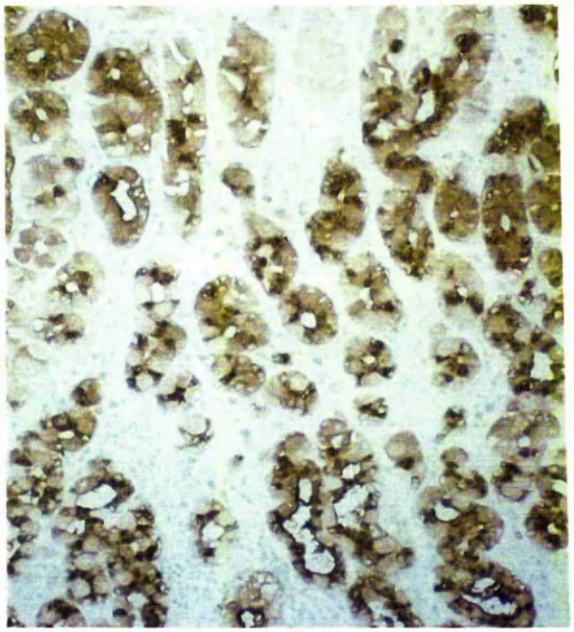

Figure 85. BSA-II on normal tissue. Chief cells were strongly stained. Parietal cells showed weak or negative staining. 400X

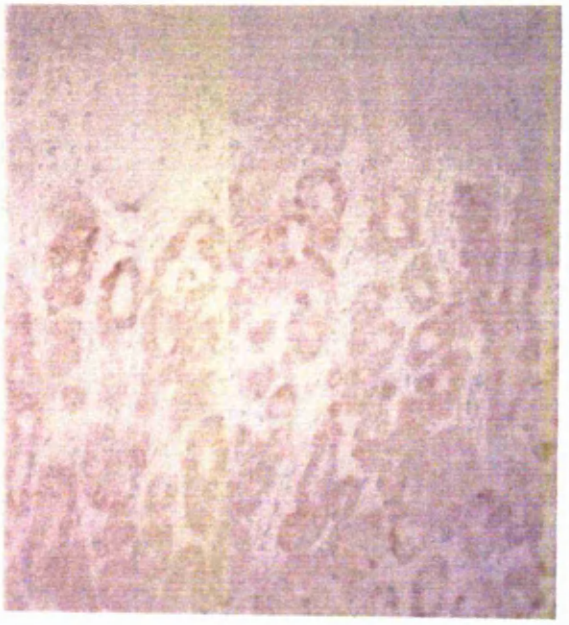

Figure 86. CA19-9 on normal tissue. Faint staining was seen in gland cells. 400X

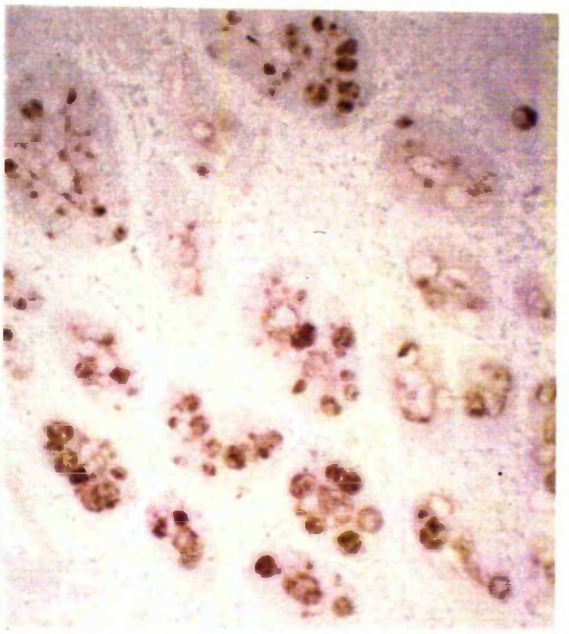

Figure 87. CA19-9 on metaplastic tissue. Epithelial cells were not stained, but the goblet cells were stained clearly. 400X

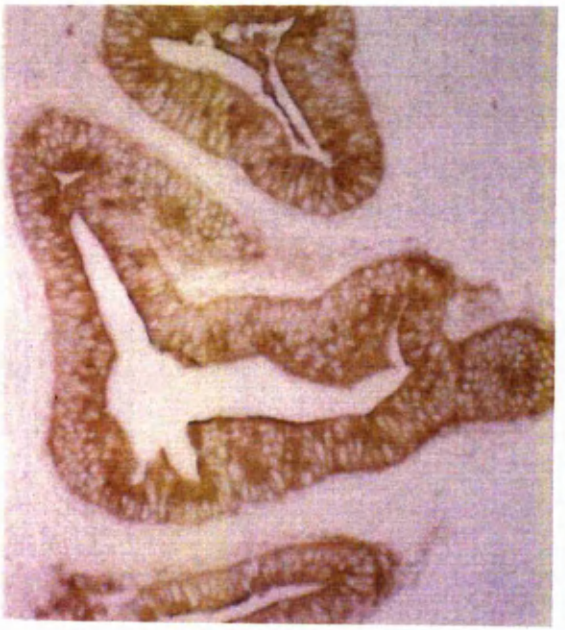

Figure 88. CA19-9 on well-differentiated tumour. Strong staining was seen in the intercellular and supranuclear regions. 800X

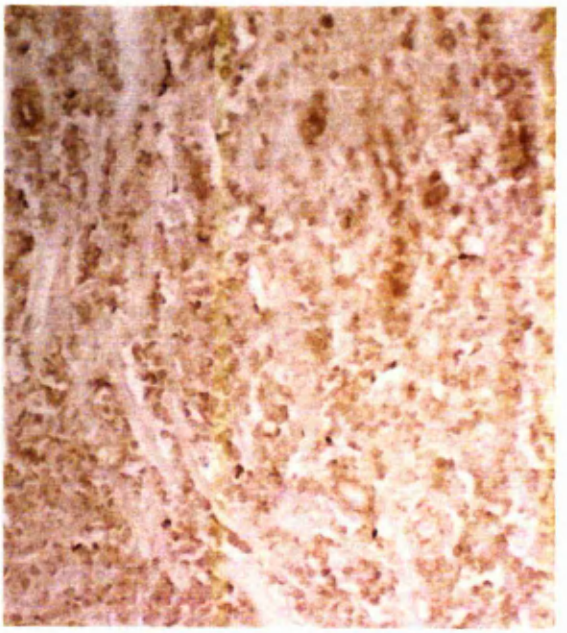

Figure 89. CA19-9 on mucinous tumour. Most cells showed cytoplasmic staining. 165X

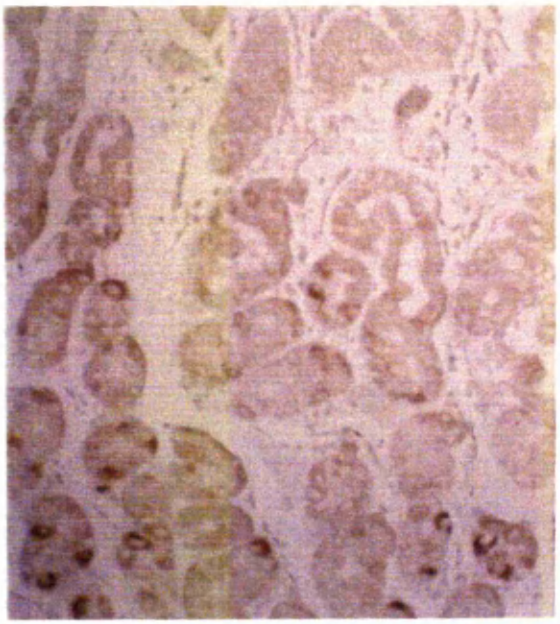

Figure 90. MUC-1 on normal tissue. Few gland cells showed perinuclear staining. 400X

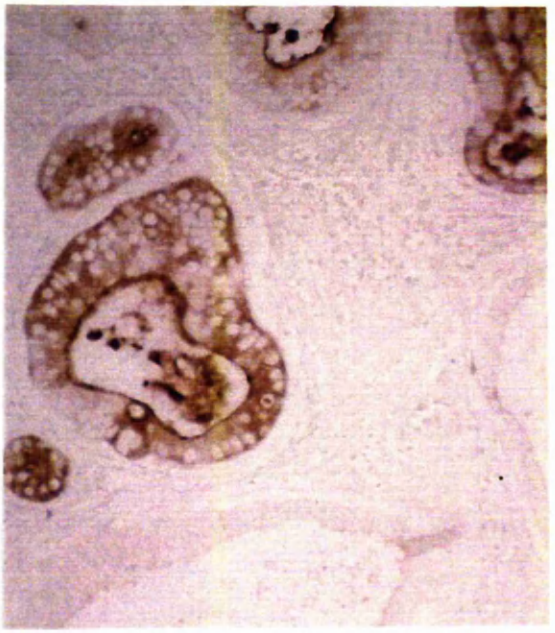

Figure 91. MUC-1 on well-differentiated tumour. Some glandular structures showed positive cells. 400X

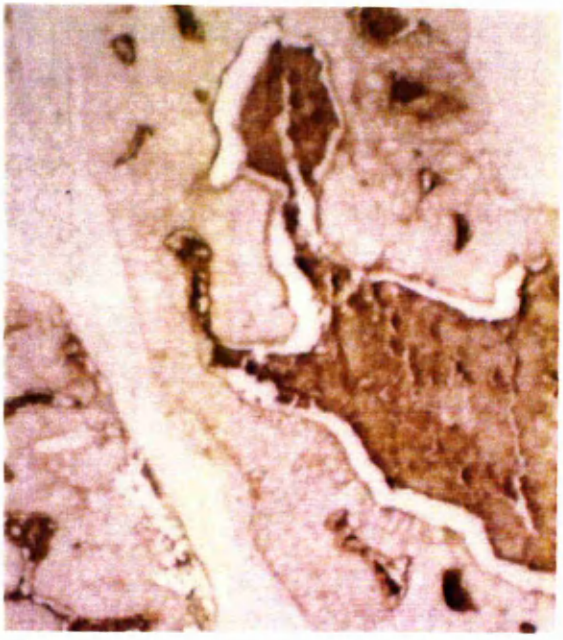

Figure 92. MUC-1 on moderately-differentiated tumour. Staining was seen in lumenal membrane regions. 800X

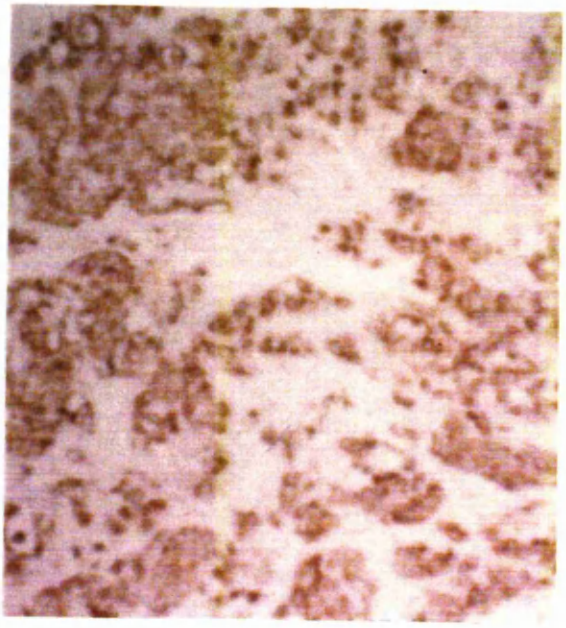

Figure 93. MUC-1 on mucinous tumour. Most cells were cytoplasmically stained. 165X

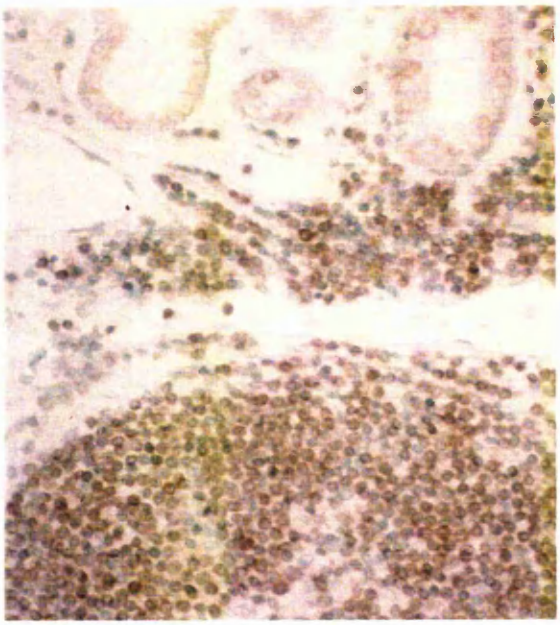

Figure 94. Bcl-2 on normal tissue. Positive staining was seen in the cytoplasm of lymphocytes and a few gland cells. 400X

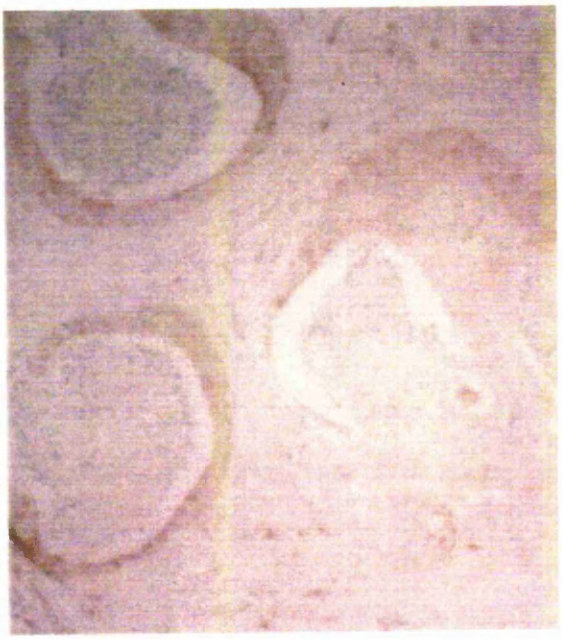

Figure 95. Bcl-2 on well-differentiated tumour.Faint staining was seen in most tumour cells. 400X

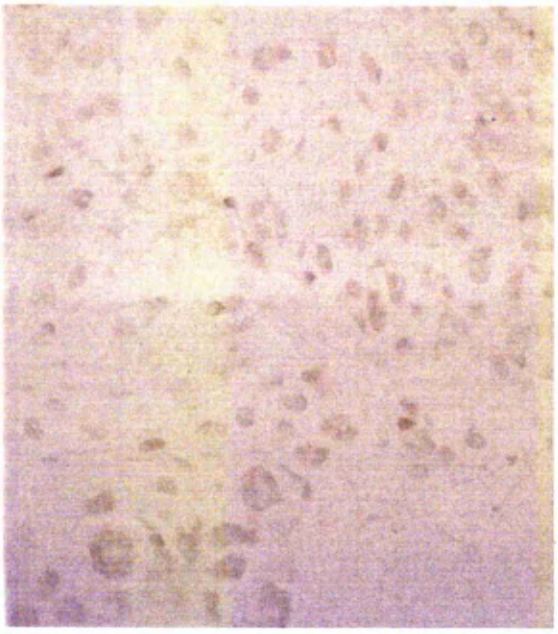

Figure 96. Bcl-2 on signet ring cell tumour. Positive staining was seen in some tumour cells. 400X

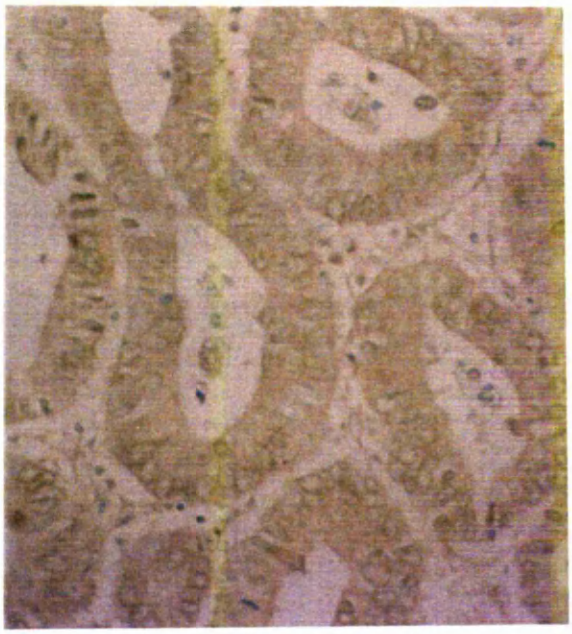

Figure 97. Ras on well-differentiated tumour. Faint staining appeared in most of cells. 400X

Figure 98. Ras on moderately-differentiated tumour. Most cells were stained intercellularly and cytoplasmically. 400X

Figure 99. Ras on signet ring cell tumour. Staining was seen intercellular membrane. 400X

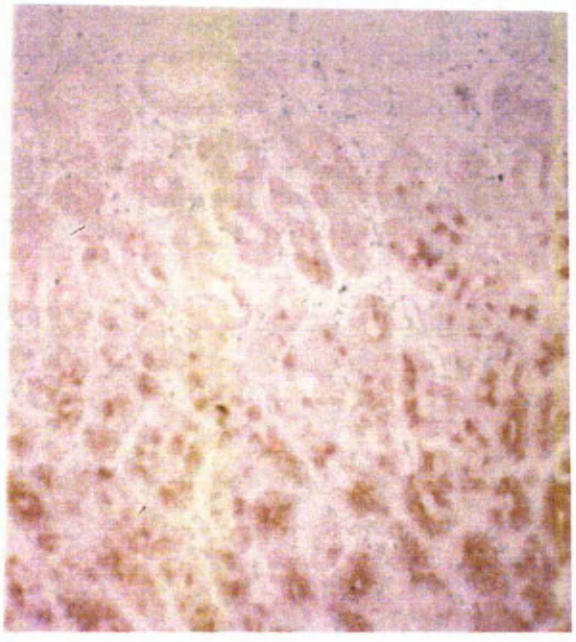

Figure 100. Rb on normal mucosa. No nuclear staining was seen except for the cytoplasmic staining in gland cells 400X

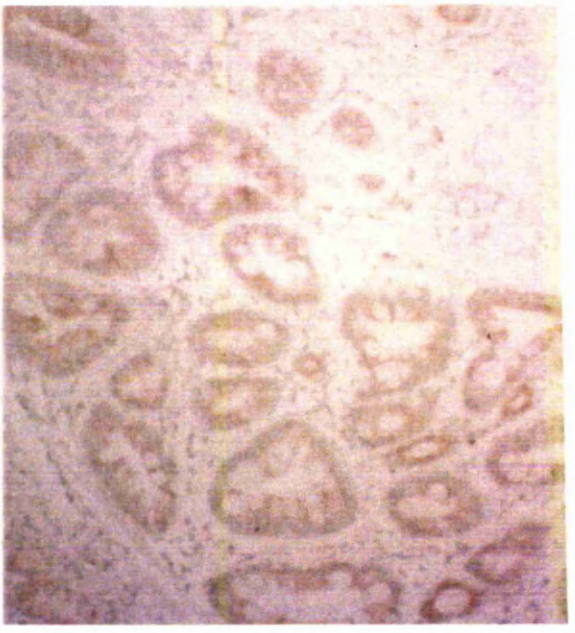

Figure 101. Rb on metaplastic tissues. Staining was seen in cytoplasm of the affected cells. 400X

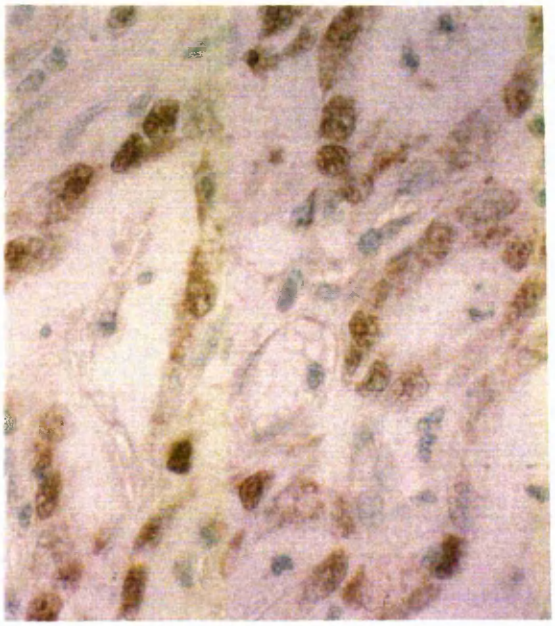

Figure102. Rb on intramucosal tumour. Most cells showed intense nuclear staining. 800X

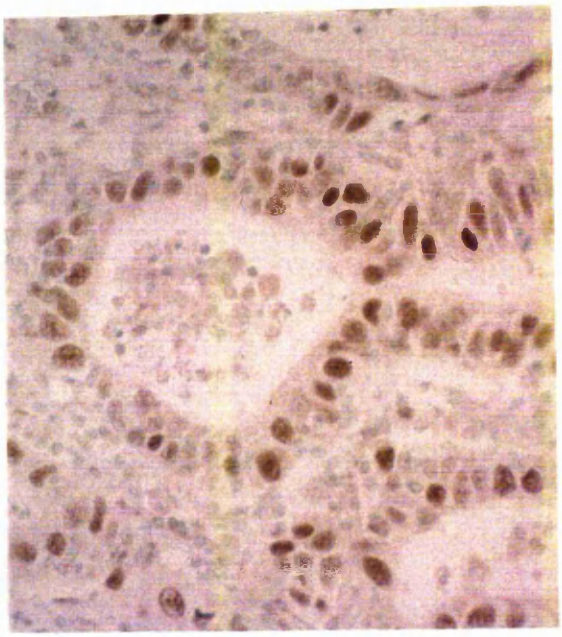

Figure 103. Rb on moderately-differentiated tumour. Nuclear staining was showed in part of tumour cells. 400X

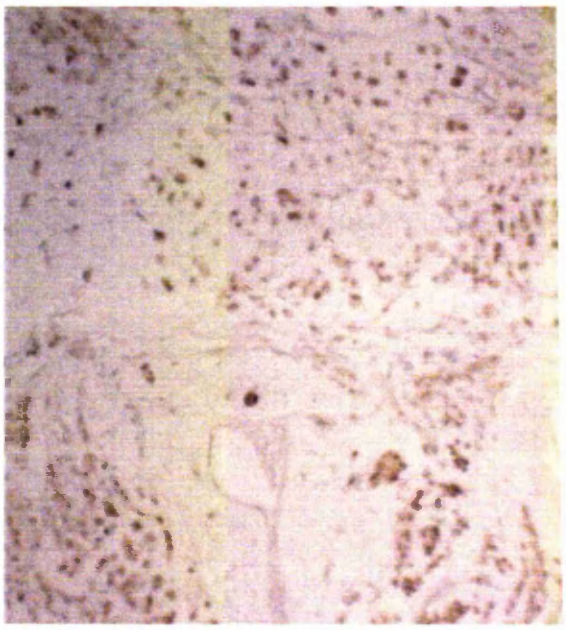

Figure 104. Rb on signet ring cell tumour. The majority of tumour cells showed positive nuclear staining. 165X

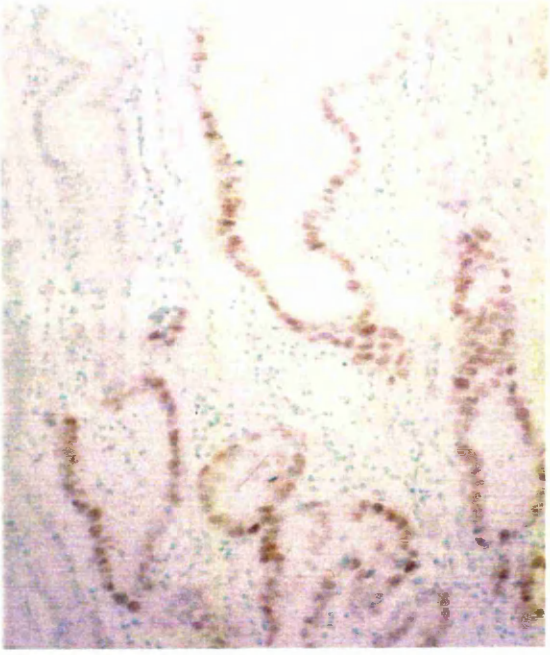

Figure 105. P53 on gastritic tissue. Hyperplastic cells showed positive nuclear staining. 400X

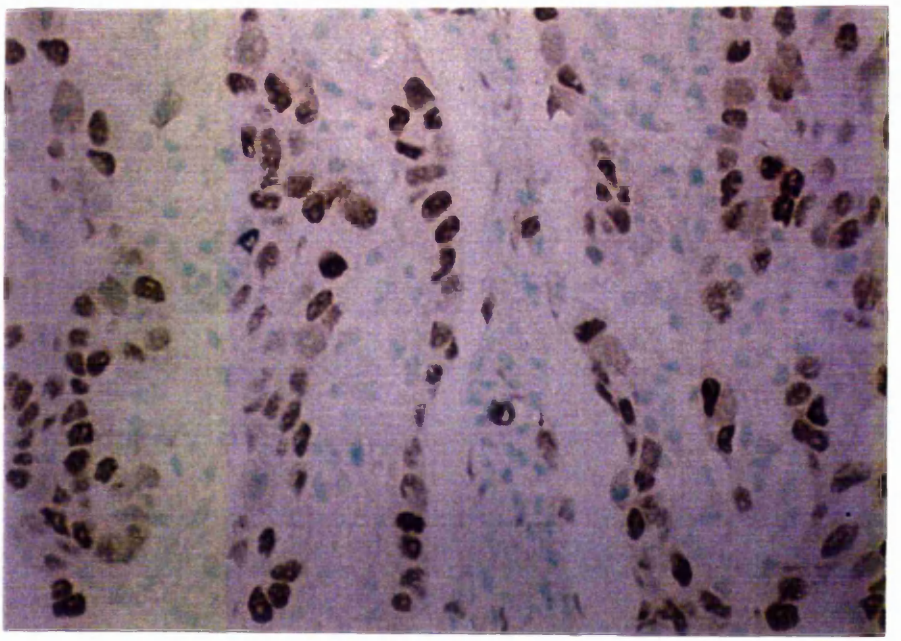

Figure 106. P53 on intramucosal tumour. Most tumour cells showed strong expression of p53 protein. 800X

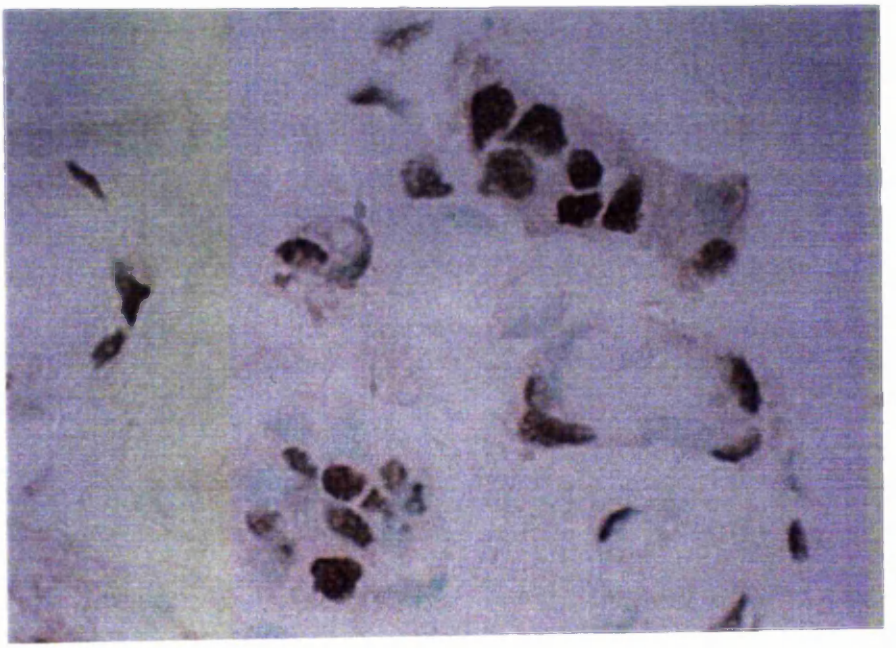

Figure107. P53 on signet ring cell tumour. Tumour cells showed staining. 1600X

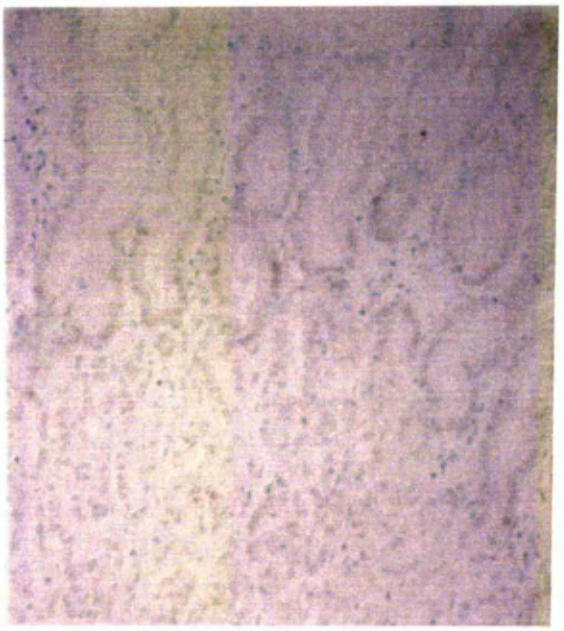

Figure 108. PCNA on normal tissue. Few positive cells in the neck zone. 165X

Figure 109. PCNA on gastritic tissue. More layers of neck cells showed positive nuclear staining. 400X

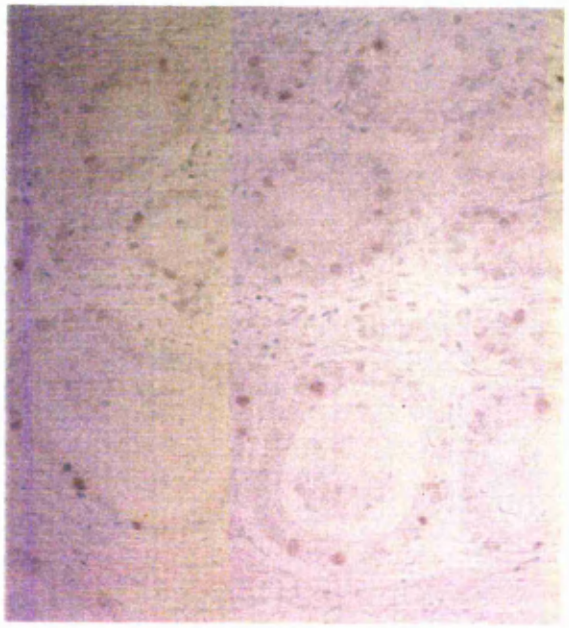

Figure 110. PCNA on well-differentiated tumour. Positive cells scattered in tumour tissue. 400X

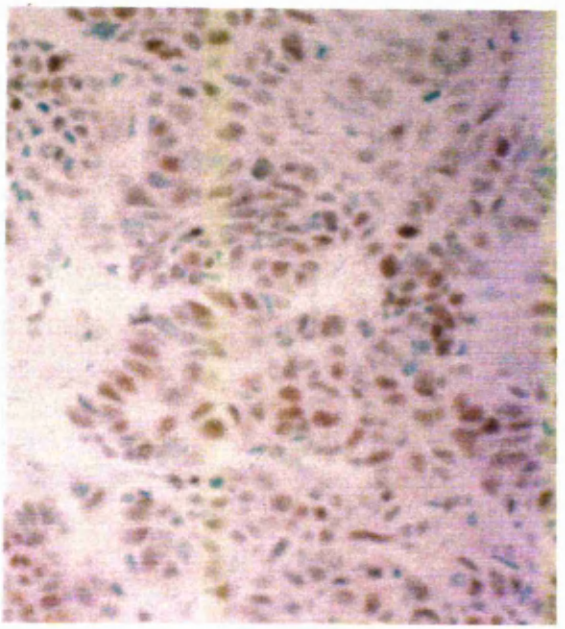

Figure 111. PCNA on mucinous tumour. A large proportion of tumour cells showed nuclear staining. 400X