The Lectins from European Mistletoe (*Viscum album*):

Isolation and detection of binding in primary breast cancer correlated with clinical behaviour.

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by

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Abstract

This thesis examines the place of **Mistletoe** plant extracts containing carbohydrate-binding proteins (lectins) in cancer treatment and their use as a tissue-based predictive test for biological behaviour of breast cancer.

It begins with an account of the **European Mistletoe** (Viscum album), its mythology, history, use as an anti-cancer drug and the isolation and identification of 3 lectins. This is followed by an account of breast cancer, its problems of management and the value of prognostic factors. A review of lectin receptors in breast cancer is included.

The practical work comprises:

- Isolation and identification of 3 mistletoe lectins by gel, ion-exchange and affinity chromatography. Purity and molecular weight were determined by polyacrylamide gel electrophoresis while major carbohydrate specificity was determined by haemagglutination inhibition. Part of this work was carried out in East Berlin/GDR.
- Raising rabbit polyclonal antibodies to the lectins. Determination of specificity by Ouchterlony gel diffusion, enzyme linked immunosorbent assay (ELISA), dot- and Western type immunoblotting.
- Using the 3 isolated lectins to stain paraffin sections of 234 primary breast cancers, by a histochemical method.
- Correlation of clinical behaviour with staining (lectin-binding) of sections, comparing histological type and size

of primary, blood group, lymph node status, disease free interval (recurrence) and survival of 234 patients followed for up to 11 years.

The lectins used showed major sugar specificities as follows:
Mistletoe lectin I(MLI): Galactose
Mistletoe lectin II(MLII): N-Acetyl-D-Galactosamine (GalNAc)
Mistletoe lectin III(MLIII): GalNAc
No significant correlation was seen between the binding of the 3 lectins to paraffin sections and histology type, grade, tumour size, blood group nor lymph node status. However, differences were seen between survival of stainers and non-stainers for each of the 3 lectins, especially MLIII, although this difference only just reached statistical significance ($P < 0.0441$).
A significant association between staining and recurrence was seen with all 3 lectins, in particular MLIII ($P < 0.0007$), Having a major specificity for the sugar GalNAc.
This result might suggest that primary breast cancers expressing this sugar are likely to metastasize sooner than cancers that do not.
Detection of mistletoe lectins binding to receptors in primary breast cancer sections, and assessment of their role in behaviour of breast cancer does not appear to have been described before.

To my parents

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1. HISTORICAL REVIEW AND LITERATURE SURVEY

1.1. Mistletoe plant (*Viscum album* L.)

Mistletoe is one of the semiparasitic plants, i.e. they depend for water and mineral nutrition on their respective host but are able to produce carbohydrates by photosynthesis (Becker, 1973). The European Mistletoe, *V. album* L., is found growing from Northern Europe to Northwest Africa, going East from Europe, through Southwest and Central Asia to Japan (Hegi, 1981). According to its host specificity, the European population is divided into subspecies (Becker, 1986).

V. album (Fig 1) has green, much branched stems, usually branched in pairs, and is evergreen. Male flowers exceed the female ones in size and both flowers bloom in Febuary, March or April. The berry-like fruit is sticky and white. The fruits are eaten by birds and the seeds are passed out of the alimentary tract thus facilitating migration of the plant among the trees (Kuijt, 1969; Beurton et al, 1989).

Fig. 1
The European Mistletoe plant (*Viscum album*)

1.1.1 History and Mythology

The European Mistletoe V. album L is one of few plants whose fame goes back into prehistory. The fame of the Mistletoe, according to Tubeuf (1923)(cited by Kuijt, 1969), is associated with the saga of the death of the favourite of gods, Balder. When Balder started to have threatening dreams, his mother, the godess Frigg, placed all objects and beings under oath not to harm him. But the evil spirit Loki had observed that Frigg neglected to take the oath for one object which was considered unimportant, because it seemed to be a part of the trees in which it grew, the Mistletoe. While the other gods amused themselves in proving that even spears and stones could not harm Balder, Loki took an arrow he had made from the wood of Mistletoe to the god Hudor, Balder's brother, who was unable to take part in the sport because of his blindness. Loki handed Hudor the arrow of Mistletoe and showed him how to fire it at Balder. When Hudor did so, his brother fell dead.

To prevent such an incident happening again, the plant was put under Frigg's control but only so long as it did not touch the earth, the empire of the evil Loki.

In later times, a sprig of Mistletoe worn round the neck was said to be a talisman against witchcraft, as long as it had never been allowed to touch the ground. This appears to be the reason why people still suspend Mistletoe above the ground today and that **"whenever persons of opposite sexes pass under it, they give one another the kiss of peace and love, in full assurance that this plant is no longer an instrument of mischief"** (Kuijt, 1969).

In the Mistletoe cult of the druids, the plant occupied a formal religious

status. According to Pliny (1923) (cited by Kuijt, 1969) and to Fraser (1900)(also cited by Kuijt, 1969), at certain times, probably at mid Summer's Eve, a sacrificial ceremony was performed. The Mistletoe, preferably growing on oak, was cut from the tree with a golden sickle by a druid, and caught in a white cloth. Only then was the sacrifice made : animals and even human beings were sacrificed.

Lindow man (Fig 2), who was killed around 300 BC and found in a peat bog in Cheshire in 1984, had grains of Mistletoe pollen in his stomach. His throat was cut and he had also been garotted with a thong.
Archeologists have suggested that Lindow man may have been killed by druids in a sacred grove in April or May while the Mistletoe was in bloom. Equally, he may have been taking Mistletoe as a potion to give himself magical strength or as an ancient remedy for epilepsy or some other illness (Brothwell, 1986) as Mistletoe preparations, have been reportedly used for thousands of years for a wide variety of therapeutic purposes; epilepsy, infertility, hypertension, connective tissue disorders and cancer (Kuijt, 1969, Franz, 1986).

1.1.2. Present use of Mistletoe preparations as an anti-cancer drug.

Mistletoe is used in various ways for medicinal purposes, as fermented preparations of the squeezed sap, or as alcoholic-aqueous extracts, sometimes in combination with other drugs (Wagner et al, 1986).

Various Mistletoe preparations (Iscador, Helixor, Plenosol), have been used in the treatment of cancer all over Europe (Wagner et al, 1986; Holtskog, et al, 1988). The oldest and best known of which is called

Fig. 2 Lindow man {Reproduced from Brothwell, 1986}

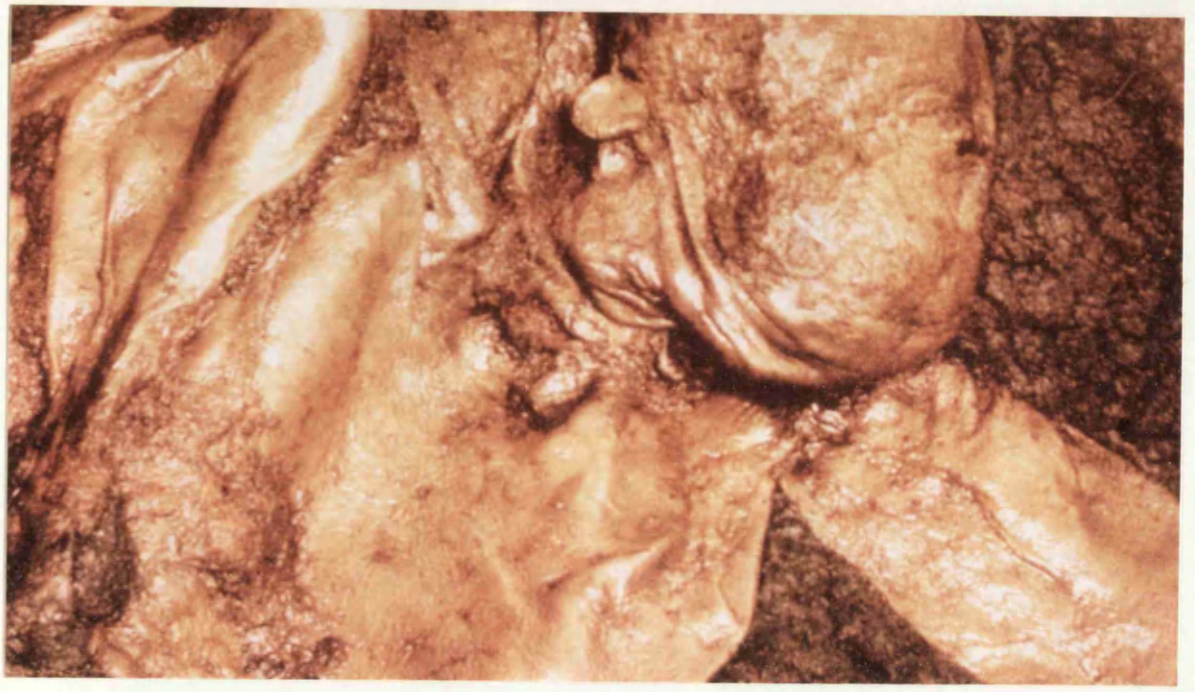


Fig. 2 Remains of an ancient human body, found in a bog of Lindow Moss, Cheshire in 1984. Apparently ritually slaughtered, he was felled with an axe, garotted with a thin "cord", and his throat was cut open.

Iscador.

In 1926, a product made from crudely pressed juice of the V. album plant called "Iscador" was introduced in Europe as an immunotherapeutic agent for cancer (Evans and Preece, 1973).

The anticancer activities of Iscador have been ascribed to a combination of cytotoxic and immunological reactions (Evans and Preece, 1973; Leroi, 1979a; Stripe et al, 1980; Olsnes et al, 1982). However, Iscador use was and still is controversial; there were reports that were concerned about its use such as the American Cancer Society report (1982) which called Iscador treatment an **"Unproven method of Cancer management"**.

The first significant impetus for the immunological research on Iscador was given by Nienhaus et al (1970). They demonstrated that repeated intraepithelial injections of Iscador in mice induced an enlargement of the thymus. This observation was confirmed 11 years later by Rentea et al (1981) who reported that repeated intraepithelial injections of Iscador caused a proliferation of lymphoid and reticulum cells in the cortex of the thymus and a doubling of the weight of the gland in 9 days.

Pleural effusions:

In 1978, Salzer and Muller, reported a great success rate of treating metastatic "carcinosis pleurae" with Iscador. Their study was based on 53 patients. Following clinical and radiological diagnosis of the effusion, fluid is withdrawn for cytological examination. Forty six Of the 53 malignant pleural effusions dried up, whereas 4 patients died with their effusion persisting. When these 53 patients were divided into groups according to the primary tumour that had given rise to the pleural effusion, they included 39 cases of breast cancer,36 of whom were "successfully treated". Salzer and Muller stated that " this difference in

behaviour is not yet explained, nor is the action of Iscador in drying up the effusion".

In 1980, Bock and Salzer, confirmed the reported effect of Iscador on malignant pleural effusions. Later in 1981, Salzer, reporting on the use of Iscador therapy for cancer, confirmed the effect of Iscador in drying up malignant effusions and described the cytomorphological appearance of treated effusions: the first fluid sample usually contained numerous tumour cells, the lymphocyte count was within normal range, and there were no eosinophils. In subsequent (treated) fluid samples, the number of tumour cells had definitely gone down, or disapeared completely. Degenerating tumour cells were also noted. At the same time, lymphocytes and eosinophils tended to show a marked increase. Lymphocytes forming a rosette around the tumour cells were not uncommon, and lymphocytes were occasionally seen invading tumour plaques. A number of effusions (7 of 62) showed no morphological changes, despite the fact that clinically the effusion had stopped.

Salzer confirmed his report of 1981 in 1986 and concluded that Iscador has two distinct activities, **cytotoxic activity** and **immunostimulating activity.**

Useful results were also reported using Iscador therapy on patients who have been surgically treated for bronchogenic and stomach cancer, on patients with very advanced stages of the disease with inoperable colorectal carcinoma , and on patients with secondary liver cancer from different primary tumour sites (digestive tract, lung and breast) (Leroi,1979b; Hoffman,1979; Salzer, 1981). Increase in survival time in terms of months was reported in these studies.

Breast Cancer and Iscador treatment:

The effect of Iscador therapy on patients with breast cancer has been investigated by Hajto (1986). In this investigation, all breast cancer patients had undergone surgical intervention (at various times) prior to the examination. Some had received chemotherapy, some received radiotherapy and some received anti-estrogens, but not within 6 months prior to the examination. A fter a single intravenous infusion of Iscador, several immunological parameters in the peripheral blood of these patients were examined. Parallel with neutrophilia, and with increase of juvenile neutrophils, a significant enhancement of phagocytic activity of granulocytes was shown. after a short decrease during the first 24 hours, a significant increase in natural killer (NK) and antibody-dependent cell-mediated cytotoxicity (ADCC) activity as well as augmented levels of large granular lymphocytes were observed. Hajto concluded , on the basis of the significant alterations in various immunological parameters, that Iscador may have an important role in the immunomodulatory therapy of cancer.

Hajto and Lanzrein (1986) confirmed that Iscador has a stimulating effect on many natural host defense mechanisms. In addition, Iscador was found to increase the synthesis of tumour necrosis factor (TNF), gamma interferon and interleukin-2 (Hajto and Hostanska, 1989).

Several proteins have been characterized in Mistletoe preparations, particularly three glycoproteins classified as lectins (Franz, 1985), four "viscotoxins" (basic polypeptides of molecular weight, MW. around 5KD) and ten other basic proteins, the so called Vester protein complex (Vester, 1977) which apparently has potent cancerostatic and immunomodulatory properties. Moreover the presence of cytotoxic alkaloids (Tasneem et al, 1986) and immunomodulatory polysaccharides (Jordan and Wagner, 1986) have been described.

Although this large number of constituents has been isolated from Mistletoe preparations, it is not yet known which compounds are responsible for its reported actions.

Hajto in 1986 has speculated that "on the basis of several recent data it appears that Mistletoe lectin and its isolated chains may be of importance in Iscador activity". Hajto 's speculation was shared with Wagner et al (1986) who related the action of Mistletoe preparations on cancer to the presence of high molecular weight compounds, lectins, viscotoxins and polysaccharides but, they speculated that in view of their cytotoxic and immunmodulatory properties, the lectins seem to be the most interesting of these groups of constituents.

1.2. Lectins

1.2.1. Definition

More than a hundred years ago (1888), Stillmark described a haemagglutinin (that he called ricin) in an extract of castor beans (Ricinus communis). Since then a large number of carbohydrate-

binding proteins have been isolated from plants and animals and studied in many ways. The name "lectin", which is derived from the Latin (legere = to choose, to pick out), was first intoduced by Boyd and Shapleigh (1954) to describe a class of proteins of plant origin which agglutinate cells and exhibit antibody-like sugar binding specificity.

Goldstein et al (1980) defined lectins as **proteins or glycoproteins that bind to carbohydrates, having at least 2 binding sites to carbohydrate, agglutinate cells and/or precipitate glycoproteins and are not formed by the immune system.**

Lectins are generally accepted to be proteins or glycoproteins with specific binding sites for sugars but are not antibodies or enzymes (Kocourek and Horejsi, 1981) and in 1982a, Franz et al redefined lectins as **proteins or glycoproteins, with one or more binding sites to carbohydrates and are not enzymes or antibodies.**

1.2.2. Main Characteristic of lectins

1- Lectins are widely distributed in nature: found predominantly in the seeds of plants, but they are also present in other parts of plants such as roots and leaves. In addition they are found in viruses, bacteria, plants, invertebrates and vertebrates (Sharon and Lis, 1972, Kocourek, 1986, Goldstein and Poretz, 1986).

2- Generally contain carbohydrate but there are some exceptions eg Con A (no carbohydrate) (Lis and Sharon 1986a).

3- The actual property common to all lectins is an ability to bind to sugars, in particular to carbohydrate components of glycoconjugates

(Table; 1). Although specific binding can be very tight, it is not covalent (Goldstein et al, 1980, Franz et al, 1982a).

Table; I
Carbohydrate specificity of some lectins
(Goldstein and Poretz, 1986)

Lectin	Sugar specificity
Concanavalin A (Con A)	Man* > Glc** > GlcNAc***
Ricinus communis I (Ricin I)	BGal> Gal**** > GalNAc*****
Abrus precatorius (abrin)	Gal > GalNAc
Arachis hypogaea (Peanut)	GalB1, 3GalNAc > and BGal
Phaseolus vulgaris	GalB1, 4GlcNAcB1, 2Man
Phytolacca americana	GlcNAc(B1, 4GlcNAc) = (GalB1, 4GlcNAc)
Lotus tetragonolobus	L-Fuc****** > L-Fuc 1, 2GalB, 4GlcNAc
Ulex europeus I	L-Fuc
Helix pomatia	GalNAc 1, 3GalNAc > GalNAc
Dolichos biflorus	GalNAc 1, 3GalNAc > GalNAc
Soy bean (Glycine max)	BGalNAc > and BGal

*Man= Mannose **Glc= Glucose *** GlcNAc= N-acetyl-galactosamine
**** Gal = Galactose ***** GalNAc = N-acetyl-galactosamine
****** Fuc = Fucose

4- Majority of lectins agglutinate red blood cells (RBCs) but are not blood group specific (Kocourek, 1986). Lectins of same carbohydrate specificity do not necessarily bind to RBCs of same blood group (Ponder, 1983).

5- Some lectins are toxic. Interestingly, the first lectin prepared by Stillmark (1888) was the highly toxic ricin, which has structural and carbohydrate binding specificity (Galactose) similar to the main lectin from Mistletoe (Lutsik, 1975).

1.2.3. Mistletoe (*Viscum album*) lectins

A lectin from *V. album* was first described by Krupe, who in 1956 found that extracts of *V. album* agglutinated RBCs. Krupe's finding was confirmed two years later by Bird (1958).

In 1970, Pardoe et al found haemagglutinating lectin in the berries of *V. album*. They described it as galactose-specific and showed it agglutinated tumour cells (of Burkitt-EB 2 lymphoma).

1.2.3.1. Isolation and identification

A common method for the isolation of lectins has been sugar affinity chromatography. Affinity chromatography to isolate Mistletoe lectin(s) was first described by Luther et al (1973), who used human B erythrocytes as a solid phase. The lectin was then liberated by heating the agglutinated RBCs (56^{o}C, 15 minutes).

In 1977, Franz et al, reported the isolation of a lectin from V. album, by affinity chromatography on insolubilized serum proteins and further fractionation by gel chromatography using Sephadex G75.

Ziska et al (1978) described a method to isolate and purify the lectin by affinity chromatography using O-lactosyl-polyacrylamide, O-galactosyl-polyacrylamide or hydrolized sepharose 4B. The lectin isolated was of a MW of 115KD, estimated by gel chromatography. The lectin exhibited a single band by polyacrylamide disc electrophoresis. A MW of 29KD and 34KD was obtained for the subunits in 10% polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS). The purified lectin possessed no specificity to human blood groups RBCs but the

haemagglutination was inhibited by lactose and D-galactose.

In 1979, Luther et al, using human B erythrocytes as a solid phase and, by elution with D-galactose, isolated a lectin that they called Viscum-lectin. They characterized the lectin as a glycoprotein containing 11% carbohydrates (glucose, mannose and N-Acetyl-D-glucosamine). The isoelectric point of the Viscum- lectin was estimated at pH 6.1, by isoelectric focusing. The lectin reacted with human and animal erythrocytes, with ascites tumour cells of mice, with human immunoglobulins and with the lectin Con A. The lectin was found to have a specificity for D-galactose. By sedimentation coefficients of 4.3-4.7 S, two different types of MWs with about 60KD and 120KD (dimerization in higher concentration) could be analyzed. By SDS-PAGE, The intact lectin showed evidence for 2 large molecules with a MW of 57KD and 55KD. In addition 3 more faint bands were seen with a MW of 33KD, 31K and 29KD. The lectin was found (by SDS- PAGE in the presence of B-mercaptoethanol) to be composed of 2 subunits with a MW of 34KD and 29KD, connected by disulphide bonds.

Franz et al (1981), by affinity chromatography isolated 3 different lectins from *V. album* using Mistletoe grown on the locust tree (*Robinia pseudoacacia*). They called the isolated lectins : Mistletoe lectin I (MLI), Mistletoe lectin II (MLII) and Mistletoe lectin III (MLIII).

MLI: 100g of the ground material was mixed with 750ml of 1.5M Nacl. After centrifugation, the supernatant was applied to a column of acid treated Sepharose. An elution using 0.2M D-galactose yielded about 40-50mg lectin.

MLII: The effluent passing through a column of partially hydrolized sepharose was put onto an immunoglobulin-Sepharose column. Corresponding elution by 0.2M galactose yielded 2-3mg of MLII.

MLIII: After displacing MLII, the 3rd lectin was eluted by glycine/HCL pH 2.6. The haemagglutinating fractions were pooled and neutralized then put on a column of Sepharose-N-(6-aminohexanl)B-D-galactosamine. The column was then eluted with glycine/HCL buffer pH 2.6 to yield 10-20mg of MLIII.

Characteristics of the 3 lectins:

The three lectins showed no blood group specificity. By polyacrylamide-gel disc electrophoresis, MLI and MLII each gave a single band, and MLIII showed a major band and three faint bands. After reduction with B-mercaptoethanol, each of the lectins showed two bands in disc PAGE in the presence of SDS. MLII , MLIII and also the subunits of MLI contained two chains linked by a disulphide bridge. **MLI** was found to be of a MW of 115KD, with 4 chains of MW of 34KD and 29KD. **MLII** of MW 60KD, has 2 chains of 32KD and 27KD. **MLIII** of a MW of 50KD, has 2 chains of MW of 30KD and 25KD. By a haemagglutination inhibition test, MLI was found to be galactose specific, MLII was found to be galactose/N-Acety-galactosamine (GalNAc) specific, MLIII was found to be GalNAc specific. MLII and MLIII cross-reacted with MLI antibody in radial immunodiffusion. Ziska and Franz (1985) and Franz (1989) confirmed the presence of 3 different lectins in *V. album* extract.

Olsnes et al (1982) isolated only 1 lectin from extracts of *V. album* grown on Norway maple (*Acer platanoides*), by affinity chromatography on acid treated Sepharose 4B. The lectin migrated in PAGE in the presence of SDS corresponding to a MW of 60KD. In addition, two bands migrating corresponding to MW of 29KD and 32KD were also found. In the presence of 2- mercaptoethanol, only two bands migrating corresponding to MW of 34KD and MW of 29KD were found. They called the isolated lectin viscumin.

Samtleben and Kiefer (1985), used an acid-treated Sepharose 4B column and elution with 0.2M lactose. The eluate was further purified by Sephadex G100 column chromatography, isolating 2 lectins. They named the lectins *V. album* agglutinin I (VAAI) and *V. album* agglutinin II (VAAII). The MW of the intact VAAI was found to be 65KD. When the agglutinin was treated with 2-mercaptoethanol at 45°C for 1 hr before electrophoresis, this protein gave rise to 2 subunits of MW of 34KD and 29KD. The MW of the intact VAAII was found to be 60KD and 58KD with 2 subunits of MW of 32KD and 27KD. The best inhibition of agglutination was found to be with D-galactose for VAAI and D-galactose and GalNAc for VAAII.

The results of isolation of Mistletoe lectins by different groups are summerized in table; 2.

Table; 2
Isolation of Mistletoe lectins

Authors	lectins	MW/ intact lectin	MW/ chains
Luther et al (1979)	Viscumin	58KD + 55KD (major) 29KD, 31KD & 33KD(minor)	34KD + 29KD
Franz et al (1981)	MLI	115KD	34KD (X2) + 29DK (X2)
	MLII	60KD	32DKD + 27DKD
	MLIII	50KD	30KD + 25KD
Olsnes et al (1982)	Viscumin	60KD (major)+ 32KD + 29KD(minor)	34KD + 29KD
Samtleben and Kiefer (1985)	VAAI	65KD	34KD + 29KD
	VAAII	60KD + 58KD	32KD+ 27KD

1.2.3.2. Antibodies to *V. album* lectins

In 1981, Franz et al, raised polyclonal antiserum to MLI in order to quantitate Mistletoe lectins in Mistletoe extract. The resulting antibodies were able to recognize the complete range of lectins. This was found by Ziska and Franz in 1985, who developed an enzyme linked immunosorbent assay (ELISA) and found that MLII and MLIII cross-reacted to 12.5% and 25% respectively with the MLI immunoassay. Thus any assay involving the use of polyclonal anti-MLI antibody may not allow differentiation between the three lectins. Franz and his co-workers have raised monoclonal antibodies to Mistletoe lectins I but even the monoclonal antibodies were able to recognize the 3 lectins (Ziska, 1989, personal communication).

1.2.3.3. Histology/Morphology of Mistletoe plant

Neumann et al (1986), demonstrated the lectins in leaves of V. album by means of gold-labelled anti- Mistletoe antibody.

Al-Alousi et al (1989a), by immunoperoxidase staining methods on formalin-fixed paraffin-embedded sections of different parts of Viscum album, using polyclonal rabbit anti-Mistletoe antibody, demonstrated the presence of lectins in leaves, stems and berries of the plant with the strongest staining reaction present in the leaves. This result was confirmed by ELISA on extracts of different parts of the plant. ELISA showed that the lectin is widely distributed in the plant with the leaves containing the largest amount (Al-Alousi and Leathem 1989a).

1.2.3.4. Mistletoe lectin I and its chains A and B

All Mistletoe lectins consist of two different types of glycoprotein chains named A and B chains. The B-chain carries the lectin activity (carbohydrate binding site), whereas the A chain is the toxic part. The majority of A and B chains are conjugated by S-S bonds (Franz, 1989b) (Fig. 3), but there exist also A and B chains held together by non-covalent bonds (Lutsch et al, 1984).

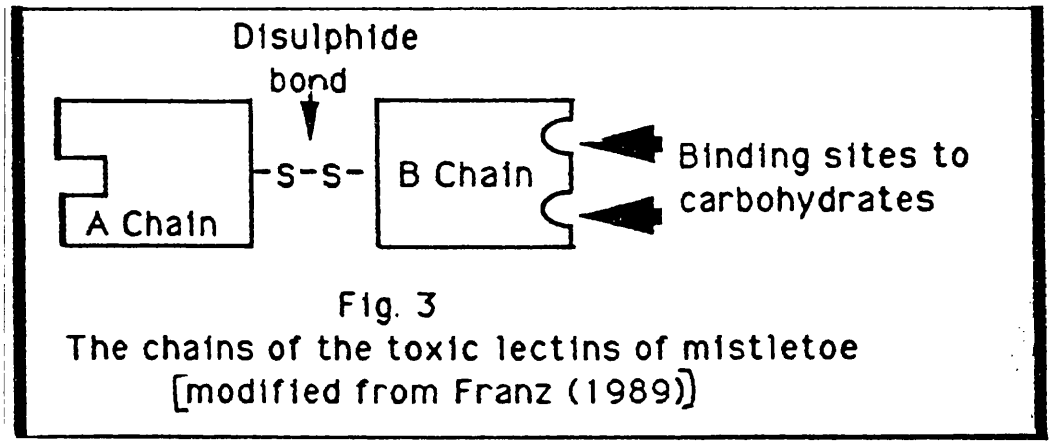


Fig. 3
The chains of the toxic lectins of mistletoe
[modified from Franz (1989)]

MLI occurs in a monomeric (AB) (MW 60,000D) and in a dimeric (AB)2 form (MW 115-120KD, A chain 29KD, B chain 34KD). Both chains are glycoproteins, which for the most part are linked by disulphide bridges and form dimers (Franz et al, 1981, Franz et al 1982b). The A and B chain of MLI were prepared under non-denaturing conditions by the use of 2-mercaptoethanol by Franz (1986).

1.2.3.4.1 Biological activity of the chains of MLI/ cytotoxicity

In 1973, Luther et al, described cytotoxic activities of a lectin from V. album, on Ehrlich mouse ascites tumour cells (EMA) and Zajdela hepatoma ascites cells (rat), eliminated after binding of the lectin to human erythrocytes.

Stirpe et al (1980, 1982) and Stirpe (1983) demonstrated a mechanism of MLI toxicity by inhibition of protein synthesis at the ribosomal level . They also studied the effect of sugars on the sensitivity of cells to the lectin. Lactose and galactose afforded some protection of cells against viscumin. Glucose, mannose and L- fucose did not protect against viscumin.

The high toxicity of MLI, by inhibiting protein synthesis at ribosomal level was confirmed by Sargiacomo and Hughes (1982) and by Samtleben and Kiefer (1985).

The application of A and B chains prepared under nondenaturing conditions enabled Franz et al (1982) and Franz et al (1983) to investigate the influence of the different chains on protein synthesis. They found that the A chain inhibits the protein synthesis significantly on the ribosomal level. The effect was strongly dose-dependent. The B-chain was without influence.

This finding was confirmed by Olsnes, et al (1982) who pointed out that this A chain acts on ribosomes even after treatment of MLI by SDS. Anti-MLI antibodies inhibited this activity.

The toxicity of MLI and its A and B chains on Ehrlich mouse ascites (EMA) tumour cells was studied at an ultrastructural level by Franz (1985) who demonstrated that 0.5µg/ml of MLI caused cell destruction and deformation of the cell organelles. Franz explained that MLI can bind to glycoconjugates on the cell surface via the B chain. This fixation (according to Franz) allows the invasion of the A chain into the cell. On the ribosomal level, the A chain inhibits protein synthesis. However , Franz suggested that the serious damage

and alterations of the EMA cells cannot be caused only by the mechanism described above and that immediate membrane alterations seem to be likely too. Franz has also found that incubation of EMA with the A chain alone does not give any alterations. The same results were also obtained using the B chain. Higher concentrations caused enlargement of mitochondria. Thus, Franz concluded that it seems to be combined A and B chains that exhibit the cytotoxic capacity.

The alteration of ribosomes leading to an inhibition of protein synthesis by the A chain makes this glycoprotein a candidate for the preparation of immunotoxin (monoclonal antibodies coupled to a toxic substance) (Franz, 1986).

1.2.3.4.2 Biological activity of the chains of MLI/ mitogenicity

Mitogenicity

Lectins are characterized by their ability to bind specifically to sugars and as a consequence to sugar containing compounds on the cell surface (Goldstein et al, 1980; Kocourek and Horejsi, 1981).

With respect to their effects on lymphoid cells, the lectins can be divided into mitogenic, indifferent and inhibitory groups (Dillner-Centerlind et al, 1980).

One of the most dramatic effects of interaction by lectins with living cells is mitogenic stimulation, i.e., the triggering of nondividing lymphocytes into a state of growth and proliferation (Lis and Sharon, 1986).

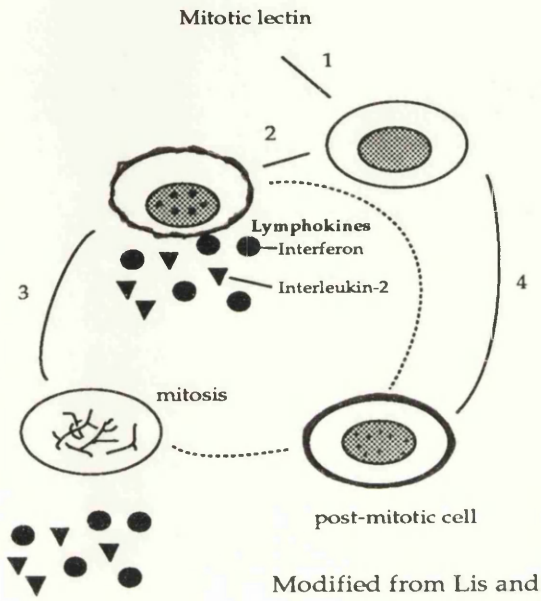
The first mitogenic agent to be described was Phytohaemagglutinin (PHA), the lectin from red kidney bean (from *Phaseolus vulgaris*) (Nowell, 1960). Later many additional mitogenic lectins were described, such as Pokeweed mitogen and Con A (Lis and Sharon, 1986).

In the mechanism of mitogenic stimulation, it is generally accepted that the initial step is binding of the lectin to cell-surface sugars. Binding alone however is not sufficient since certain lectins are nonmitogenic (e.g., HPA) even though they bind human lymphocytes (Dillner-Centerlind et al, 1980).

Essentially all metabolic processes in mitogen-treated lymphocytes are stimulated, though to varying degrees, and at different times after exposure to the mitogen:
First membrane changes. Later RNA and protein synthesis accelerate and morphological changes become apparent. Finally DNA synthesis starts and the cells enter mitosis. This is followed by the reversion of the postmitotic cells to small lymphocytes resembling those originally stimulated or, if the mitogen remains in the medium, restimulation of some of the cells through the proliferative cycle (Lis and Sharon, 1986).

Accumulating evidence indicates that binding of mitogenic lectins to T lymphocytes activate lymphocytes to release a variety of biologically active polypeptides, known as lymphokines, the best characterized of which are interleukin-2 (Robb, 1984) and interferon (Rinderknecht et al, 1984).
Events in mitogenic stimulation are illustrated below in Fig. 4.

Fig. 4 Events in mitogenic stimulation.
-Membrane changes, lymphokine production, increase cell size, morphological changes.
-Undergoing mitosis, continued lymphokine production.
-Revision to resting lymphocyte.
-Recycle with continued presence of mitogen.



Modified from Lis and Sharon 1986

In 1985, Franz, in an investigation on the biological activities of MLI and its A and B chains, studying their effect on mononuclear cell cultures, reported surprisingly that the A chain of MLI acted as a mitogen. The A chain stimulated lymphocytes at concentrations of 0.1μg and 1 $\mu g/10^6$ cells. However, the intact MLI and the B chain showed a strong inhibitory effect on mitosis which disappeared with decreasing concentrations of MLI and the B chain. Franz concluded that MLI and the isolated B chain belong to the inhibitory lectins.

Franz's observation was confirmed by Metzner et al in 1986 who evaluated the mitogenic activity of MLI and its A and B chains on cultures of mononuclear cells taken from peripheral blood of 25 healthy donors. They found that MLI and isolated B chain act as inhibitory lectins and that the A chain acts as a mitogenic. They stated that **" the mitogenic activity of the A chain could be one reason for immunostimulating effects of Mistletoe preparations in cancer therapy".**

1.2.3.4.3 Other biological activities of the chains of MLI

In addition to the above mentioned biological activities of MLI and its A and B chain, MLI and its B chain were found to be capable of immediate activation of macrophages (Franz, 1986). In addition , increasing leukocyte phagocytosis was also ascribed to the B chain only. Besides the immediate binding, the B-chain can also indirectly activate macrophages by release of lymphokines (e.g. macrophage stimulating factor) from lymphocytes (Metzner et al, 1987). Thus, the B-chain both inhibits the proliferation of lymphocytes and liberates lymphokines from the same cells.

Hajto et al (1989) isolated and purified MLI from Iscador and found that the lectin and its B chain to increase, significantly, NK cytotoxicity and the level of large granular lymphocytes (LGL) in peripheral blood of rabbits and breast cancer patients, when injected intravenously in optimized dose (not toxic dose).

The stimulating effect of MLI on many natural host defence mechanisms was confirmed by Hajto and Hostanska (1989).

1.2.3.5. MLII and MLIII

Most studies on the biological activities have concentrated on MLI and its A and B chains. MLII and MLIII do not appear to have been studied yet.

Wagner et al (1986), determined quantitively the lectin content in Iscador and in fermented Mistletoe extract, by single radial immunodiffusion. As shown by the isoelecric focusing method, Iscador and fermented Mistletoe extracts contained only the Mistletoe lectins that Franz et al in 1981 described and called MLII and MLIII and the complex that Samtleben and Kiefer (1985) called VAII, whereas the proteins of the MLI (VAAI) complex were missing. They suggested that the typical MLI proteins are probably completely degraded during the fermentation procedure used in the production of Mistletoe extract.

A rapid decrease of lectin concentration during fermentation was also reported by Ribereau-Gayon et al (1986a). They found, by ELISA, that bacterially fermented Iscador contained a low amount of lectins, approximately 100ng/ml, while unfermented Iscador contained 10 times more. A low amount of lectins in bacterially fermented Mistletoe extract has also been reported by Ziska and Franz (1985), using ELISA.

Olsnes et al (1982) reported that a product in Mistletoe extract, possibly identical to MLIII/MLIII, had only 1% of the toxicity of MLI. This finding led Wagner et al (1986) to speculate that this could explain the high tolerance to fermented Mistletoe preparations when they are

administrated parenterally!

Doser (1985) (cited by Franz, 1986), studied the effect of a partially purified lectin he obtained commercially from Sigma on a culture of human T leukemia cell line Molt 4. He found this lectin to have a high toxic activity when added to the culture medium. The cytotoxic effect of this lectin was significantly diminished by addition of GalNAc, whereas galactose was without any inhibitive effect. Franz (1986), who cited this study, speculated that perhaps the cytotoxicity of this lectin preparation was mainly due to MLII and/or MLIII which according to Wagner et al (1986) are the essential lectins in Iscador.

The presence in Iscador of a toxic substance that is identical with MLII described by Franz et al (1981), VAAII described by Samtleben and Kiefer (1985), was also the conclusion reached by Holtskog et al (1988), who have also observed the absence of MLI (VAAI) from Iscador preparations.

When injected intravenously, MLII and MLIII were found to have the same effect as that of MLI in increasing NK cytotoxicity and increasing the level of LGL in peripheral blood of rabbits and breast cancer patients but the effect was weaker than that of MLI (Hajto and Hostanska, 1989).

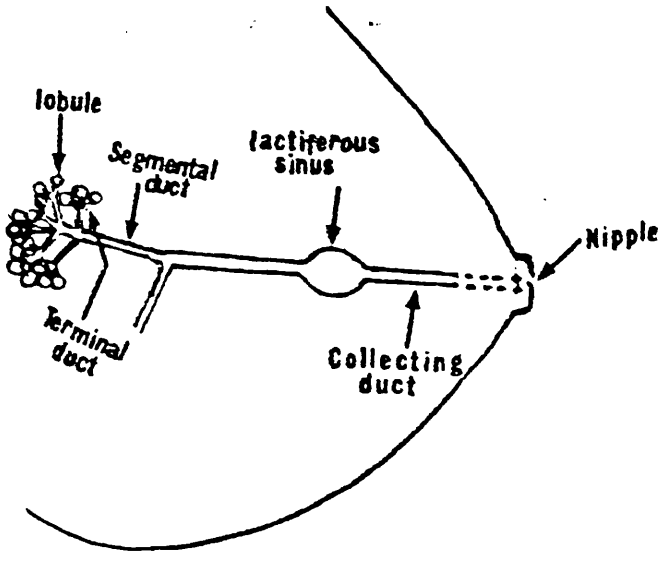
1.3. Breast

1.3.1. Breast structure

The breast is a secretory gland which consists of epithelial cells of alveoli and ducts plus their supporting muscular and fascial elements and varying amounts of fat, blood vessels, nerves and lymphatics (Haagensen, 1986).

The epithelial component is made up of 20 or more lobes, each emptying into separate excretory ducts terminating at the nipple. The lobes, are divided into a multitude of lobules, each made up of from 10-100 or more alveoli grouped around a collecting duct. As the main collecting ducts enter the base of the nipple, they enlarge to form the milk sinuses which dilate and act as a reservoir for milk in nursing. The numbers and size of the lobules vary exceedingly , and not always according to the development. Each secretory duct, of which there are about 20, with its secondary and tertiary ducts and accompanying alveolae represents a lobe (Sloane, 1985; Haagensen, 1986) (Fig. 5).

Fig. 5
Ductal and lobular system of female breast



Breast growth is largely dependent upon estrogen and progesterone (Wile and DiSaia, 1989). In studies utilizing oophorectomized animals, estrogen replacement stimulates ductal growth whereas progesterone is necessary for adequate alveolar growth (Jimerson, 1981). However, full differentiation of the gland requires insulin, cortisol, thyroxine, prolactin, and growth hormone (Speroff et al, 1983). Changes occur regularly in response to the estrogen-progesterone sequence of a normal menstrual cycle (Wile and DiSaia, 1989).

1.3.2. Breast cancer

The term cancer refers to an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues. Unlike most benign growth, the cells in the malignant growth invade the surrounding tissues (local invasion) and have the ability to spread to distant sites (metastasize) (Anderson, 1982). It is recognized that breast cancers in individual patients may differ widely from one another in natural history and response to treatment (Haagensen, 1986). Breast cancer is the most common malignancy in women worldwide. In Britain 1 women in 10 is likely to develop breast cancer at sometime during her life time (Spratt et al, 1988). In 1984, 21,363 women were registered as having breast cancer in England and Wales (Cancer statistics registration, 1984), and the incidence continues to rise and at present (1989) increases by 2% per year (Chamberlain, 1989).

In 1987, In England and Wales, 13,751 women died of breast cancer (Mortality Statistics Cause, 1987). The latest figures show that the mortality rate in the United Kingdom from breast cancer is the highest in the world varying from 29.44 to 37.50/100,000 in different parts of Britain (Boyle et al, 1989). The mortality rate for similar European or USA

populations is 27/100,000 or less, and across the world goes down to 5 for Japan, 0.73 for Thailand or even 0.07 for Nicaragua (Segi, 1981).

1.3.2.1. Breast cancer metastasis

The major clinical problems of breast cancer are caused by the extension of the disease locally and/or its widespread dissemination. During the last decade , it has become clear that the major determinant of outcome in breast cancer is the existence of metastasis at the time of diagnosis (Holmberg et al, 1989).

Metastasis is the establishment of a new, "secondary" tumour by a cancer cell that travels through the bloodstream from a "primary" tumour. (Fidler and Hart, 1982; Feldman and Eisenbach, 1988)

Metastasis is the main clinical problem of cancer. Usually by the time the "primary" tumour is detected cells have already escaped from it and settled in other organs, sowing the seeds for "secondary" tumours there, thus begins a race against malignancy that the patient rarely wins (Feldman and Eisenbach, 1988).

Although a minority of cancers display such behaviour (metastasis), the fact it occurs at all has led to some interesting hypotheses on its causes (Nicolson, 1988a).

1.3.2.1.1. Theories on Metastasis with special reference to breast cancer metastasis

In breast cancer several studies have been attributed to understanding the relationship between the primary cancer in the breast, its spread to draining LN(s) and its spread to distant sites.

(1) Halstead's concept of the spread of breast cancer (1907) (cited by Hellman et al,1982) was of interest. He believed that all extensions of the cancer from the primary were by **direct spread**. This concept of the spread of the disease was the basis for performing radical resection of the affected area. However it is not until the 1930s when ideas changed to think that 'embolization' was the predominant mode of spread to draining LN and to distant sites (Gray, 1939).

(2) Another theory of breast cancer metastasis that provided the basis for treatment through the first half of this century was that the spread of breast cancer is **sequential: arising in the breast, spreading first to regional nodes, and finally to distant sites.** According to this view, the regional LN(s) act as a filter or barrier to the further spread of the cancer. Later it was recognized that the axillary LNs do not function as the principal source of cells that spread to distant sites and that spread to LN(s) and to distant sites represent two independent (but correlated) processes (Carlson and Stockdale, 1988).
However, very recently Hartveit (1989) illustrated the process of spread to regional lymph node describing it as progressive (a diagram modified from Hartveit ,1989, is shown below in Fig. 6).

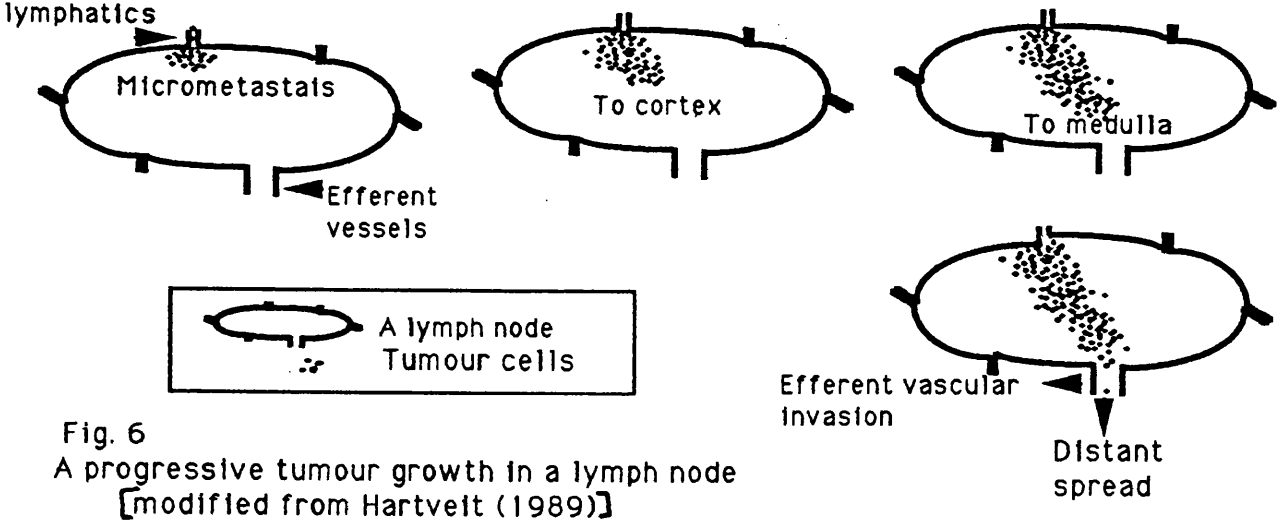


Fig. 6
A progressive tumour growth in a lymph node [modified from Hartveit (1989)]

Hartveit suggests that the spread of breast tumour cells first to regional lymph nodes and finally to distant sites is one mechanism by which breast cancer disseminate. According to him, tumour cells enter the nodes via the afferent lymphatics and leave them by the larger efferent trunk.

(3) Ewing (1928) (cited by Nicolson, 1988a, 1988b) proposed that metastasis formation (in general, not only breast cancer) at regional and distant sites is primarily due to lymphatic and circulatory anatomy and the mechanical lodgment of malignant cells at the first barrier encountered. A frequent distant organ site of blood-borne metastatic involvement is lung, consistent with the **Ewing's "anatomical-mechanical"** theory of cancer metastasis. Almost all evidence for regional tumour spread tends to support anatomical or mechanical mechanisms (Sugarbaker, 1981; Nicolson, 1988a, 1988b).

Malignant cells circulating in the blood from the venous circulation should arrive initially in the lungs and lodge in their microcirculation. The eventual formation of metastases, however, often occurs in organs distant from the initial organ encountered (Nicolson, 1988a, 1988b). In some cancers, unique organ colonization patterns may be found that do not fit the"anatomical-mechanical" model for cancer dissemination

(Sugarbaker, 1979; Miller et al, 1985; Nicolson, 1988a, 1988b), eg. kidney, bronchus.

Breast carcinomas metastasize to bone, brain and liver in most patients with metastatic disease, along with the expected high incidence of metastasis to lung (Bonadonna et al, 1976).

Anatomical-mechanical hypotheses do not explain the non-random distributions and high frequencies of brain, liver and bone metastases from metastatic breast cancer (Einhorn et al, 1974; Bonadonna et al, 1976; Nicolson, 1988a, 1988b).

(4) To explain metastatic colonization patterns that cannot be due to mechanical lodgment and anatomical considerations, **Paget (1889)** proposed the **"seed and soil"** hypothesis, which proposed that metastatic development was a consequence of particular or specialized tumour cells (seeds) finding a suitable environment (soil) in order to develop and grow. This hypothesis attributed to various tissues different metabolic or biological properties that could either enhance or inhibit the growth of arrested tumour cells. Paget (1889) (cited by Nicolson, 1988a, 1988b) developed his hypothesis from observations on the dissemination of microorganisms and breast cancer cells in patients; he found that the spleen was a common site of bacterial abcesses but not of breast cancer colonies. He concluded that spleen was not more frequently involved by metastases because it was not a favourable site for the survival and growth of cancer cells that circulated to the spleen. The other esential element was that the cancer cells that lodged in the spleen apparently did not have the correct properties for survival and growth at this unique site.

Do data from experimental animal metastasis systems support the " anatomical-mechanical" or "seed-soil" hypothesis?

The use of appropriate metastatic tumour models, such as B16 melanoma (Fidler, 1976), has resulted in greater understanding of the metastasis process but also in equivocal support of either of these two theories for tumour dissemenination. According to Nicolson (1988a, 1988b): **both** the anatomical-mechanical and seed-soil hypotheses contribute to the observed patterns of distant metastatic spread, but their individual importance can vary widely in different tumour systems.

The formation of metastases in many tumour systems is not governed solely by the passive filtration and mechanical arrest of tumour cells in the first capillary system ecncountered.
Zeidman and Buss (1952) in a classic experiment observed that circulating tumour cells or their multicell emboli were not always arrested in the first capillary bed, these **cells could be deformed and released to recirculate to other organs.**

Tarin and Price (1981) concluded that simple mechanical or anatomical factors could not explain the colonization patterns seen after intravenous injection of mammary tumour cells into syngeneic animals. they found that the ability to colonize particular organ sites appeared to be determined by the intrinsic properties of the tumour cells themselves and of the organ site involved.

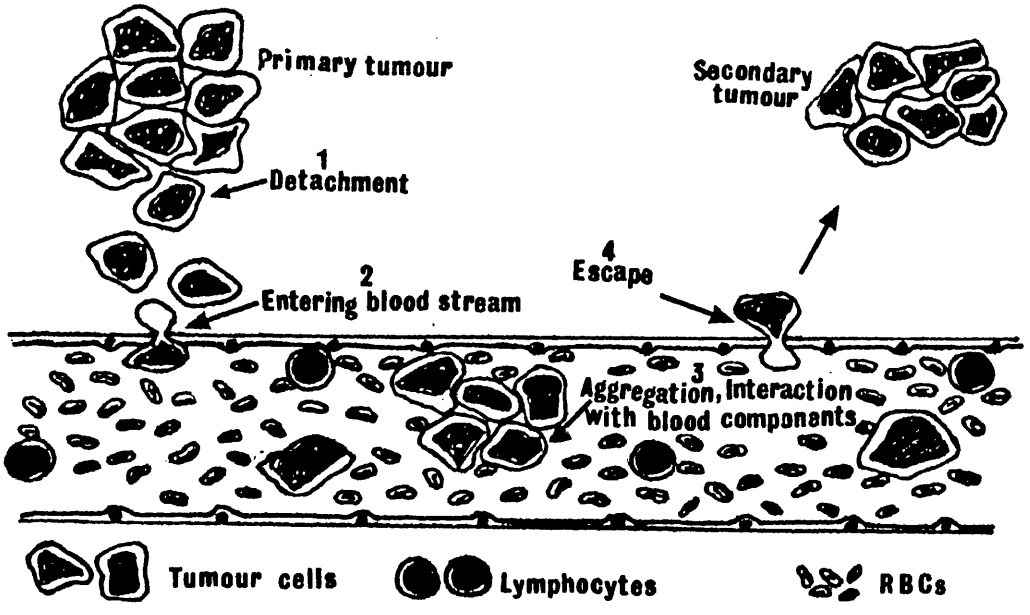
(5) An alternative explanation for the appearance of metastases at sites that do not have a direct venous circulatory connection with the primary tumour was put forward by **Bross and Blumenson (1976)** who proposed that organs become sequentially involved by "stepwise" metastatic spread.

This concept of sequential organ involvement or **"metastatic cascade"** proposes that cancers first metastasize to a "generalized site" and then from these sites they further disseminate to other sites. This has possibly received support by Hartveit (1989).

1.3.2.1.2. The process of metastasis

That metastases form by way of a complex series of sequential steps involving specific tumour cell and host properties is now widely accepted (Nicolson, 1988a, 1988b). The metastasizing cell must first **(a) detach** from the primary tumour **(b) invade the matrix** surrounding the primary tumour and then **(c) penetrate** blood-vessel walls to enter the circulation. In the blood-stream, the cell seeks out a suitable location for the formation of a new tumour; along the way it **(d) interacts** with platelets, lymphocytes and other blood components and it may aggregate with other metastasizing tumour cells. When the cell reaches a target site, **(e) it escapes from the blood vessel** and lodges in the adjacent tissue. There **(f) it proliferates** (Feldman andEisenbach, 1988) (a diagram showing the steps of metastasis modified from Feldman and Eisenbach, 1988, is shown below in Fig. 7).

Fig. 7 The process of metastasis



Only few cells could or would survive the process owing to tumour cell death in each compartment during tumour dissemination (Weiss, 1988). **But even one single tumour cell could be enough to yield metastasis** (Fidler, 1976).

Whether a tumour will metastasize is determined by the ability of tumour cells to invade host tissue barriers, then to the ability of circulating tumour cells to survive, evade possible host defence mechanisms, attach firmly to the endothelium of distant small vessels, gain entrance to the extravascular region, and to proliferate as a metastatic colony (Nicolson, 1988a, 1988b; Liotta, 1988a; Feldman and Eisenbach, 1988; Liotta, 1989).

(1) Detachment and release into blood stream

Detachment of tumour cells from the primary neoplasm is one of the first events in the metastatic process, in which there are several critical steps that can be affected by tumour growth, necrosis, degradative enzymes, and adhesion (Weiss, 1983).
Data have strongly supported ideas that the cell surface plays a critical role in the ability of tumour cells to detach and metastasize (Nicolson, 1984, 1988a, 1988b; Feldman and Eisenbach, 1988).

(2) Tumour cell-endothelial cells interaction

The adhesion of circulating malignant cells to specific organ microvessel endothelial cells is probably one of the more important events in determining organ-specific metastasis (Nicolson, 1988a, 1988b).

Some of the organ endothelial cell surface components important in tumour cell-endothelial cell interactions have been identified and been shown to display organ-specific determinants (Belloni et al, 1986; Nicolson, 1988a, 1988b). A variety of data implicate a role of cell surface glycoconjugates and of endogenous lectins {carbohydrate receptors that could be demonstrated by using antibodies against the native lectin (Mermomsky et al, 1986)} in important steps of the metastatic process. Specific changes in glycoconjugates on malignant cells are closely associated with metastasis formation in some metastatic systems. Tumour cell metastatic and endothelial cell-binding properties can be affected by metabolic alterations in tumour cell surface oligosacchariudes (Nicolson, 1988a, 1988b; Dennis and Laferte, 1989).

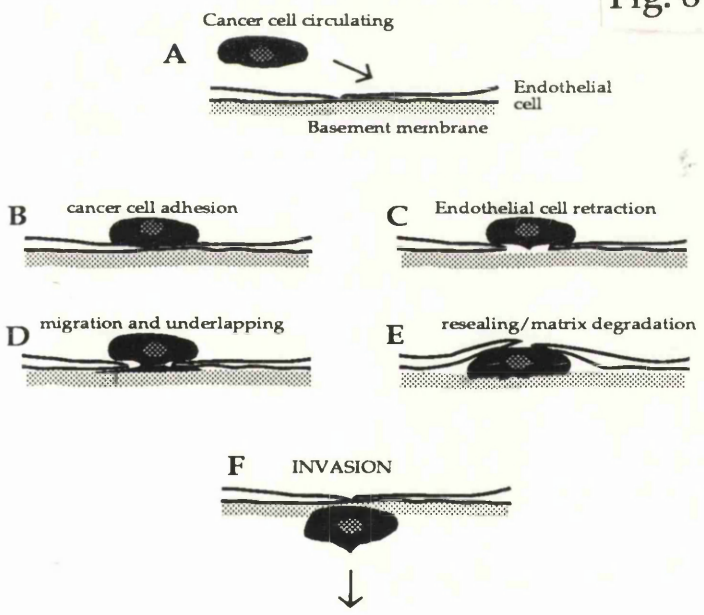
Several adhesion systems may be responsible for organ-specific localization (Nicolson, 1988a, 1988b). Evidence for parallel, multiple adhesion mechanisms in tumour cell-endothelial cell interactions suggests that a variety of multiple adhesion molecules exist on various malignant cells. These include: Cell adhesion molecules (CAMs) (Brackenbury, 1985, Brackerbury and Edelman, 1988), proteoglycans (Turely, 1984), endogenous lectins (Monsigny et al, 1983; Lotan, 1987), glycolipids (McGuire et al, 1984; Cheresh et al, 1987), glycoproteins (Narita, 1989) and other molecules (Nicholson, 1988a, 1988b). Although tumour cell-endothelial cell adhesion may be important in determining the organ localization of metastases, malignant cells can also bind to the subendothelial basement membrane (Sindelar et al, 1975).

(3) Invading the target tissue

The selective invasion of malignant cells into particular tissues is

another step in the metastatic process. After the tumour cell-endothelial cell interactions have taken place, the malignant cells can stimulate endothelial cell retraction to expose the underlying basement membrane (Kramer and Nicolson, 1979; Kramer et al, 1980). Since the exposed subendothelial matrix is usually a much better adhesive substrate for tumour cells than the endothelial cell surface (Kramer et al, 1980), there is a net movement of the malignant cells to the subendothelial matrix (Kramer et al, 1980). Eventually the malignant cells underlap adjacent endothelial cells, spread on the subendothelial matrix and solubilize this matrix using a variety of degradative enzymes (Liotta et al, 1983; Nicolson, 1982) (The process of invasion is illustrated below in Fig. 8, modified from Nicolson, 1989).

Fig. 8



Not all metastatic cells use this pathway of endothelial cell and basement membrane invasion; some malignant cells arrest in the microcirculation and grow expansively until they rupture the vessel wall (Nicolson, 1989).

The invasive behaviours of malignant cells involve a number of

properties, including cell adhesion, motility, destruction of host tissues and growth (Hart, 1981). Malignant cells that have the correct set of these properties can selectively invade certain host tissues, resulting in non random invasion of tumour cells into particular tissue and organ compartments.

Like the adhesion of metastatic cells to microvessel endothelial cells, the adhesion of malignant cells to basement membranes appears to involve multiple, parallel adhesion mechanisms.

Several basement membrane components have been identified as tumour cell adhesion molecules: fibronectin (Kramer et al, 1980), laminin (Liotta, 1983; Murray et al, 1980), type IV collagen (Murray et al, 1980) and other molecules (Nicolson, 1988a, 1988b).

Using direct cell binding to the isolated basement membrane components and their experimental metastasis model, Terranova et al (1983) and Liotta et al (1986) found that the metastatic properties of breast cancers correlated with the amounts of unoccupied cell-surface laminin receptor.

Metastatic cells can also bind to extracellular matrix components that are associated with but not part of the basement membrane, such as elastin (Netland and Zetter, 1986)

(4) Growth of metastatic cells at secondary site

The growth of metastatic cells at secondary sites is also a determinant in the organ preference of metastasis (Nicolson, 1989).
The discovery that tumour cells can synthesize and secrete their own

functionally active growth factors (autocrine growth factors) (Sporn and Todaro, 1980; Salomon and Perroteau, 1986) has aided explaining the ability of some malignant cells to proliferate in a variety of tissue compartments without regard to the usual concentrations of growth factors and inhibitors that may differentially regulate normal cell growth, also in explaining the ability of certain malignant cells to proliferate at apparently the same rate in many different tissue compartments (Nicolson, 1988a, 1988b).

Growth factors, such as epidermal growth factors (EGF) and insulin-like growth factors (IGF) were isolated and characterized before their discovery as autocrine growth factors. Many of these are related by their structure or receptors to known oncogene products. In some tumours, overexpression of oncogenes that encode "autocrine" growth factors or their receptors can lead to enhanced malignancy such as the overexpression of the cerb-B-2 protein, caused by gene amplification, correlates with poor prognosis and malignant progression of breast cancer (Slamon et al, 1987).

NK cells:

In some organs, specialized normal cells may be directly involved in limiting the growth of tumour cells. For example, the Kupffer cells of the liver have known cytotoxic activities against tumour cells (Pulford and Souhami, 1985).

Roos et al (1978) noted that tumour cells in the liver were often associated with Kupffer cells, and in some cases, Kupffer cells were observed to phagocytose tumour cells.

Kupffer cells and NK cells have been reported to have anti-tumour activities (Malter et al, 1986; Price et al, 1986; Gardner et al, 1987). Not all investigations, however, support a role for NK cells in preventing tumour growth (Fodstad et al, 1984).

The ability of malignant cells to metastasize to certain organs may be influenced by their abilities to circumvent host antitumour responses. In several animal tumour metastatic systems the most malignant cells showed increased resistance to NK (Hanna, 1982; Joshi et al, 1987).

Tumour cells selected for NK resistance in vitro were found to be more metastatic in vivo (Hanna and Fidler, 1981), and suppression of host NK activities is reported to result in enhancement of metastasis formation (Hanna and Fidler, 1981).

Another important host anti-tumour response mechanism that kills neoplastic cells or prevents their growth is mediated by activated macrophages (Fidler, 1985).

(5) Tumour progression

As cancers progress, multi-site metastases are the most common finding, even in cancers that initially appear to spread to only one distant site. Thus during the malignant progression of cancers, they appear to acquire the capability of colonizing additional organ sites. These different secondary tumours might be composed of malignant cell subpopulations capable of metastasizing to different specific sites (Nicolson, 1982, 1983). Alternatively, as these tumours progress, their malignant cells may acquire additional properties that allow them to colonize many organ sites simultaneously (Nicolson, 1987). This could be due to "changes" in the malignant cell population, a phenomenon known clasically as **"tumour progression" (Foulds, 1975)** that allow further sites to be colonized.

The ability of tumours to change with time was examined by Foulds

(1956), who studied this phenomenon in mouse mammary tumours. He noted that the tumours changed gradually and independently and eventually gained autonomy from host controls that regulate normal cell growth and differentiation. Extension of malignant cells to additional metastatic sites could also be accomplished by an increase in production of tumour autocrine factors, such as autocrine growth factors, that make them more autonomous of the specific growth controls present in different tissues, and autocrine motility factors, that stimulate cell movement (Nicolson, 1988a, 1988b).

An autocrine motility factor has been isolated from metastatic tumour cells (Liotta et al, 1986). This factor, which stimulates random cell movements, was found to be synthesized in large amounts by highly metastatic mammary adenocarcinoma cells (Nicolson, 1989).

1.3.2.2. Breast cancer : Prognostic factors

Clinical and basic science investigations of breast cancer have identified a number of general biological factors and principles that aid prediction of prognosis, disease course, and benefit from therapy and are described in details below. These biological factors, which are independent but interrelated, include: Histopathology, axillary lymph node (LN) status, primary tumour size, proliferative rate and ploidy, hormone receptor status, oncogenes, growth factors, and carbohydrate expressions as detected by lectins.

(1) Histopathology

Histopathological examination of the breast cancer specimen establishes the diagnosis and also contributes prognostic information (Bloom and Richardson, 1957).

Various histological scoring systems have been described, the best known of which is the Bloom and Richardson grade (1957).

Histological type:

The histological sub-type of breast cancer is itself of prognostic value. The currently frequently accepted histological typing of breast tumours is the WHO typing (Scarff and Torloni, 1968).

Most breast cancers are "infiltrating ductal" and among the different subtypes one of particular importance, especially related to mammogram detected tumours, is the non-invasive type (intraduct carcinoma and intralobular carcinoma). These are breast tumours in which the epithelium is neoplastic, but the neoplasm has not extended through the basement memebrane of the ductal-alveolar units to invade the stroma of the breast (Haagensen, 1986).

The **invasive carcinoma** type include: ductal, lobular, medullary, tubular, papillary, mucous, squamous cell carcinoma, cribriform, Paget's disease of the breast and other rare and mixed histologies (Sloane, 1985). In these invasive tumours, the neoplastic cells have invaded through the basement membrane and infiltrate the stroma of the gland. It is this behaviour that distinguishes infiltrating carcinomas from in-situ carcinomas and accounts for the marked difference in their prognosis (Carlson and Stockdale, 1988).

Invasive ductal carcinomas are by far the most common duct tumours, accounting for almost 70% of breast cancers (Carlson and Stockdale, 1988). Invasive ductal and lobular carcinoma have the poorest prognosis (Harris et al, 1985; Dixon et al, 1985).

More differentiated cancers such as tubular carcinoma and mucinous or colloid carcinoma are relatively uncommon and carry a relatively good prognosis with regard to relapse and survival (Gallager, 1984; Dixon et

al, 1985). These invasive cancers can be further classified by histological grade, with strong correlation to survival (as below).

Histological grade:

Bloom and Richardson (1957) graded breast cancer by degree of tubule formation, mitoses and hyperchromasia, and irregularity of size, shape, and staining of nuclei into three grades; grade I had a good prognosis, while grade III was associated with a poor outcome.

Freeman et al (1979), analyzed 1,759 patients with infiltrating duct carcinoma followed over 10 years, showing statistical differences in survival at 10 years between grade I (56%) and grade III (33%).

Davis et al (1986) followed the WHO criteria which consists of a numerical coded system evaluating mitotic grade besides degree of tubule formation and nuclear pleomorphism. Grading primary breast tumours into 3 grades, they found that "tumour grade", no matter how assessed, significantly affected the 5-year relapse- free and total survival rates. The 5-year relapse free rates varied significantly between the tumour grades. The results 5 year survival and disease free interval results of Davis et al (1986) are tabulated below (Table: 3) where DFI = disease free interval, GI = grade I, GII = grade II and GIII = grade III.

Table: 3

5-yr DFI and Survival of patients with primary breast cancer of different tumour grades (Davis et al, 1986).

	No.	GI	GII	GIII	p-value
DFI					
all patients	1537	64%	51%	42%	< 0.0001
Pre/perimenopause	786	73%	61%	46%	< 0.0001
postmenopause	751	55%	40%	37%	< 0.002
Survival					
all patients	1537	86%	70%	57%	< 0.0001
pre/perimenopause	786	93%	77%	62%	< 0.0001
postmenopause	751	80%	63%	51%	< 0.0001

The overall results strongly indicate that grade 1 tumours exhibit the best prognosis while grade III tumours are associated with a more aggressive clinical course (Olszewski et al, 1981; Moran et al, 1984; Kamby et al, 1988; Howell and Millis, 1988; Contesso et al, 1989).

The relationship between tumour grade (differentiation) and cancer prognosis has been studied but despite development of a number of histological classifications, a definite system, which is broadly acceptable, applicable and reproducible has not yet been developed (Coulson et al, (1984).

(2) Proliferative rate and Ploidy

Classical pathological features associated with more aggressive behaviour of a malignancy are a high mitotic rate and nuclear anaplastic features (Saez et al, 1989). Since these characteristics are very difficult to quantitate reproducibly between different pathologists, some investigators have utilized the thymidine-labeling index (TLI) and aneuploidy (degree of abnormal DNA content) as equivalent and more quantifiable properties (Contesso et al, 1989).

The TLI enables the identification of S-phase cells. The higher the labeling index the poorer the prognosis (Benner et al, 1988).

High labeling indices occurred in tumours with high mitotic rates, high nuclear grade, and high histologic grade; particularly in premenopausal women and in estrogen receptor negative tumours (Tubiana and Koscielny, 1988).

DNA flow cytometry (FCM) is a new approach for measuring proliferative rate by identifying S-phase cells with the use of DNA-specific fluorescent stains (Rosen et al, 1987). An important advantage of this technique over TLI, is that the analysis can be performed on fixed paraffin-embedded blocks of tumour tissue (Saez et al, 1989).
McDivitt et al (1985) were able to show a good correlation between S-phase, as determined by TLI, and FCM. This finding paved the way to using FCM as the method of choice to study tumour kinetics.

FCM also offers the advantage of determining cell populations with abnormal DNA content (aneuploid). Even though most aneuploid cells contain excess DNA, aneuploidy also refers to cells that have less than

the diploid amount of DNA.
Using FCM it has been shown that the majority of tumours have aneuploid DNA populations (Saez et al, 1989).

At a median follow-up of 3.5 years. 44% of aneuploid tumours vs 23% of diploid tumours had relapsed (Coulsonet al, 1984). High indices of aneuploidy and S-phase cells in invasive breast cancers are associated with poorly differentiated and estrogen-receptor negative breast cancers and with a high incidence of distant metastases (Tubiana and Koscienly, 1988)

(3) Lymph node (LN) status

Spread of tumour cells from a primary breast carcinoma to the axillary nodes has long been recognized as indicative of poor prognosis (Harrington, 1929).
The axillary node region is the major regional lymph node drainage for carcinoma of the breast and for most of this century, the prognosis of patients with primary breast cancer has been related to the presence or absence of axillary lymph node involvement.

Parl et al (1984) estimated that patients with nodal metastasis were at 2.8 times the risk of recurrence compared to patients without metastasis.

In a study by Klintenberg et al (1985), the 5-year disease free interval for patients with LN-ve was 83%, but fell to 57% if positive nodes were present. At 10 yrs, 25% to 28% of women with LN-ve relapsed, compared with 75% of women with LN+ve (Fisher et al, 1975; Valagussa et al, 1978) (table: 4). Overall survival rates, similarly, were 65% to 82% at 10 yrs for women with LN-ve and 25% to 40% for those

with LN+ve (Fisher et al, 1975; Valagussa et al, 1978) (table, 5).

Table: 4

DFI of patients with primary breast cancer as reported by the authors acknowledged

	5-yr DFI	10-year DFI	
	Klintenberg	*Fisher*	*Valagussa*
LN-ve	83%	75%	75%
LN+ve	57%	25%	28%

Table: 5

Survival rates of patients with primary breast cancer as reported by the authors acknowledged

	Haagensen		*Fisher*	*Valagussa*
	(5-yr)	(10-yr)	(10-yr)	(10-yr)
LN-ve	87%	74%	65%	82%
LN+ve	67%	51%	25%	40%

The 5-year average of survival probability reported by Haagensen (1986) for patients with unilateral carcinoma who had **no** lymph node involvement was 87%, but for those with node involvement was 67%. The 10-year survival for those with **no** node involvement was 74% and for those with node involvement was 51% (Table; 5).

Number of involved lymph nodes:

In 1969, Fisher et al, reported an important observation that the rate of relapse is progressive with increasing number of involved axillary

nodes, in particular, if four or more nodes contained tumour, prognosis was markedly worse than if fewer were involved.

Saez et al (1989) also reported a direct correlation between axillary node status, disease-free survival and overall survival in primary breast cancer of over 5,900 cases followed-up for a median of 40 months. In 1983, in a large study, Fisher et al demonstrated that both disease free interval and overall survival worsen with increasing number of positive lymph nodes. Patients with LN-ve had an 85.4% disease free interval and 82.8% survival at 5 yrs. As the number of positive lymph nodes increased, both disease free interval and overall survival decreased; patients with one positive lymph node, for example, had a disease free interval rate of 63.6% and a survival rate of 80%, which decreased to 16.4% for disease free interval and to 28.4% for survival in patients with more than 13 positive lymph nodes.

The importance of axillary LN metastases in the prognosis of breast cancer patients led to the belief that axillary LNs served as tumour cell filters preventing the process of extension. Supporting this belief was the observation that tumours of increasing size have an increasing likelihood of axillary LN involvement (Carlson and Stockdale , 1988; Sacre, 1989).

(4) Primary tumour size

The greatest diameter of the primary tumour size is a measure of stage and can be related to the absence/presence of metastatic growth the axillary nodes (Haagensen, 1986). As Haagensen (1986) reported, less than one - third of his patients whose primary measured less than 3 cm

in diameter had axillary metastases, compared to about one-half of those with tumours over 3 cm.
Hartveit (1989). reported a clear statistically significant association between tumour diameter and nodal status (P<.0005). Primary breast cancers of increasing size exhibit an increasing likelihood for axillary lymph node metastasis. Tumours less than 1 cm in size having a likelihood of axillary lymph node metastases of 22%, while tumours greater than 6 cm in size having a liklihood of 63% (35).

Since the presence or absence of axillary lymph node metastases is predictive of the likely presence of systemic micrometastases, it is not surprising that tumours of increasing size carry a worsening prognosis (Valagussa et al, 1978). Therefore, it is unexpected that tumour size seems to be an independent prognostic determinant only in those patients with axillary LN involvement.

Tumour size appears to be a weak prognostic factor in patients in whom the axillary LNs are not involved (Valagussa, et al, 1978; Crowe et al, 1982). Thus tumours that attain a large size without exhibiting axillary LN metastasis are also unlikely to exhibit early systemic metastasis (Carlson and Stockdalel, 1988).

(5) Hormone receptors

The potential relationship between hormones and breast cancer was first realized by Beatson in 1896 (cited by Wile and Disia, 1989) when regressions were induced in advanced cancer after oophorectomy. The relationship was further strengthened with the observation that breast cancer in pregnant women carried an especially bad prognosis (Kilgor and Bloodgood, 1929).

Estrogen and most steroid hormones act on target cells that contain cytoplasmic or nuclear protein (receptors) that bind them (Carlson and Stockdale, 1988). Since receptors for estrogen (ER) are detectable in about 50% of breast cancer specimens, their determination has been used to predict responsiveness to endocrine therapy in patients with metastatic disease (McGuire, 1978).

It has been generally accepted that tumours lacking estrogen receptor (ER) seldom respond to endocrine therapy, while 60% of ER positive tumours do respond. Further, the probability of response in ER positive tumours is proportional to the quantitative amount of ER in the tumour specimen (McGuire, 1978).

Receptors for steroid hormones have an impact on prognosis in predicting not only a probable response to hormonal manipulation, but also the course of the disease.

Knight, Livingston, Gregory, McGuire (1977) were the first investigators to determine whether the presence of ER receptors would be of prognostic value in patients with breast cancer. They measured ER in a series of 145 patients with operable breast cancer and found that irrespective of age, LN status, or size of primary tumour, patients with ER-negative tumours had a higher rate of recurrence.
This finding of Knight et al, was confirmed by most subsequent studies (Cooke, George, and Griffiths, 1980; Godolphin et al, 1981). Furthermore, it was demonstrated that patients with ER-negative tumours had shorter survivals (Hahnel et al, 1979; Osborne et al, 1980; Furmanski et al, 1980).

Although the association of ER-negative with poor prognosis has

been confirmed in many studies, the finding has not been universal (Hilf et al, 1980; Howat et al, 1985)

ER does not separate histological types, but lobular carcinomas associate with higher ER than other types, whereas medullary tumours are often poor in ER (McCarty et al, 1980; Mohammed et al, 1986).
In infiltrating ductal carcinoma, decreased histological grade and increased nuclear grade, both suggestive of higher differentiation, correlate with increased ER (Millis, 1980; Fisher et al, 1981).

The interest in progesterone receptors arose at a time when investigators were looking for other factors, in addition to ER, to correlate with response to endocrine therapy; progesterone receptors (PgR) seemed ideal since estrogen stimulates its production in normal reproductive tissue and also in human breast cancer cell lines (Saez et al, 1989).

As predicted, Osborne et al (1980), found that very few tumours that are ER-negative are PgR-positive, consistent with PgR synthesis being estrogen dependent. They found that tumours with both receptors had the highest response rate to endocrine therapy while those with neither had the least. Osborne et al, have also suggested that PgR might be a better indicator than ER content.

Clark et al (1983) have also addressed this issue when they retrospectively analyzed PgR and ER in 189 patients with stage II breast cancer receiving adjuvant therapy. When evaluating disease free survival (DFS) by univariate analysis, number of positive axillary nodes, size of primary, ER, and PgR were all correlated with time to recurrence. When the relation between receptor levels and DFS was looked at, there was good correlation with both receptors, although stronger with PgR. Thus, patients with high levels of PgR had longer DFS than patients with low

levels. To evaluate the association between the two receptors, four groups according to the ER/PgR content were chosen : 1) ER+/PgR+, 2) ER+/PgR-, 3) ER-/PgR+, and 4) ER-/PgR-. In this analysis, DFS was better for the ER+/PgR+ than any of other groups. Thus these results suggested that PgR may be more important than the ER. Furthermore, when DFS was analyzed by multivariate analysis, only the number of positive axillary nodes and PgR retained significance. However, when overall survival was considered, axillary nodes and ER were more significant than PgR.

Chevallier et al (1988) have also indicated that PgR is more important than ER.

(6) Oncogenes

Oncogenes are families of genes that are conserved in evolution and that are important to regulation of normal cell growth and development. In human cells these genes are refered to as proto-oncogenes (Carlson and Stockdale, 1988).

The activation of proto-oncogenes appears to lead to neoplasia by a variety of mechanisms, including gene ampflication, point mutation, chromosomal rearrangement, or by the insertion of the DNA of the promoter of another gene in the vicinity of a proto-oncogene (Theillet et al, 1986; Slamone et al, 1987).

Abnormalities of several oncogenes are now known to exist in some, but not all, breast cancers; the presence of proto-oncogenes c-myc, c-erbB-2 (also called HER-2/neu) or the allelic deletion of the proto-oncogenes H-ras-1, c-ras-Ha, and c-myb appeared to identify breast cancers that were particularly aggressive and likely to recur over a short time period (Ohuchi et al, 1986; Escot et al, 1986; Theillet et al, 1986; Cline

et al 1987; Varley et al, 1987; Varley et al, 1987; Barnes, 1989).

The amplification of c-erb-B-2 in human adenocarcinoma was reported in 1986 by Yokoto et al. Slamon et al (1987) reported that this gene is amplified in 30% of carcinomas of the breast and that its amplification was associated with a poor prognosis.

In the same year Venter et al (1987) reported that the c-erb-B-2 amplification was associated with increased formation of the c-erb-B-2 protein, using frozen tissue sections, by immunohistochemical methods, and that the gene was amplified in 33% (12 of 36) human breast tumours.

Berger et al (1988), correlated the expression of c-erb-B-2 gene, detected in paraffin-embedded human breast cancer sections of 47 cases, using specific antiserum to this product of this gene, with parameters used in breast cancer prognosis. A statistically significant correlation was found between positive staining and tumours showing a poor nuclear grade ($P<0.02$) and with positive LN status of the patient ($P<0.02$). However, Barnes et al (1988) and Gusterson et al (1988) found no significant correlation between the expression of the product of this gene and LN status.

The amplification of cerb-B-2 (HER-2/neu) was reported to be significantly correlated with the stage of the disease but not with age, tumour size or hormone receptor positivity (Seshadri et al, 1989). However, Barnes et al (1988) have found a correlation ($p = 0.04$) between expression of gene product and tumour grade.

Some studies have found a significant relation between the expression of c-erb-B-2 protein in formalin-fixed paraffin-embedded tissues of primary breast cancer and poor outcome of the disease in respect to disease free

survival and overall survival (Lovekin et al, 1989; Wright et al, 1989). c-erb-B-2 is a gene which encodes a protein which has close structural relation to epidermal growth factor receptor (Coussens et al, 1985; Goustin et al, 1986).

(7) Growth factors

Growth factors (GFs) are polypeptides that play a part in normal growth of cells (Sainsbury et al, 1988).

The observation that tumour cell lines produce growth factors in response to estrogen stimulation (Dickson and Lippman, 1987) and that tumour cell lines have receptors for GFs including Epidermal growth factor (EGF) receptors (Real et al, 1986; Dickson and Lippman, 1987) and insulin-like growth factors (IGF) receptors (Furlanetto and DiCarlo, 1984) raised the question of a possible relationship between steroid receptors and GF receptors.

Pekonin et al (1988) measured the level of EGF receptor and IGF receptor in breast cancer tissue compared to the content of ER and PgR. EGF receptor correlated negatively to the ER and PgR ($P< 0.001$), whereas the IGF receptor correlated positively to ER and PgR ($P< 0.001$). In contrast no correlation was found between EGF receptor and IGF receptor.

The observation of an inverse relation between EGF receptors and steroid receptors have been reported by many authors (Sainsbury et al, 1985; Fitzpatrick et al, 1984).

The finding that the product of c-erb-B-2 gene was similar with EGF receptors (Coussens, et al, 1985; Goustin et al, 1986) led to much

speculation about their role in carcinogenesis. However, measurement of EGF receptor in several series of human tumours, in which the expression of c-erb-B-2 protein was measured immunocytochemically, showed no significant correlation between the two (Gusterson et al, 1987; Barnes et al, 1988; Wright et al, 1989).

Recently, the content of EGF receptors has been reported to correlate to metastases and with differentiation of primary breast cancer tumours (Santon et al, 1986).
Pekonen et al (1988) observed a higher EGF receptor binding in tumours with low histological differentiation than in tumours with high differentiation. In contrast Walker and Campeljohn (1986) found no correlation between tumour differentiation and EGF receptor. Similarly, no correlation between EGF receptor and differentiation was reported by Skoog et al (1986).

The presence of EGF receptor as an indicator of poor prognosis in patients with breast cancer was reported by Sainsbury et al (1985, 1988), who reported that tumours with a higher EGF binding have a higher potential for metastases and that metastases have a higher EGF receptor binding. However, Pekonen et al (1988) found EGF receptor binding in metastases similar to that in the primary breast tumours, and found no correlation between EGF receptor binding in breast tumours and the presence of metastases at primary operation.

The presence of IGF receptor in breast cancer (Pekonen, et al 1988) and their presence in breast tumour cell lines (Furlanetto et al, 1984), as well as the production of IGF by breast tumours (Dickson et al, 1985) suggest a role for IGF in breast tumour growth. When differentiation of ductal carcinomas was compared to IGF binding, no trend for increased

receptor binding with decreasing differentiation was evident (Pekonen et al, 1988).

(8) Carbohydrate expression

Recently, interest has been directed to study carbohydrate expression on cancer cells in a search for lectins that could be used to detect markers of clinical behaviour in breast cancer. Lectins, with their high affinity and specificity for certain carbohydrate residues, have been used in histochemistry as tools to detect sugar labels (Damjanov, 1987).

(8.1.) Carbohydrate structure

The basic monosacharide carbohydrate structure is a six-carbon ring or chain with L and D configurations.

Of hundreds of such sugar groups which exist, only about 7 are commonly found in mammals; they are: mannosyl-(Man), glucosyl-(Glc), galactosyl-(Gal) (Fig. 9A), fucosyl-(Fuc), N-acetyl-galactosyl-(GalNAc) (Fig. 9B), N- acetyl-glucosaminyl- (GlcNAc), and sialic acid groups, but the specificity conferred by sugars results from their arrangement in chains (Lehninger, 1970).

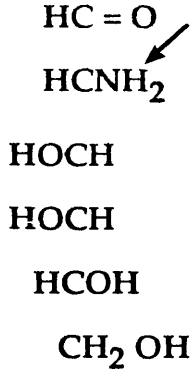


Fig. 9a: N-Acetryl-galactosamine (GalNAc)

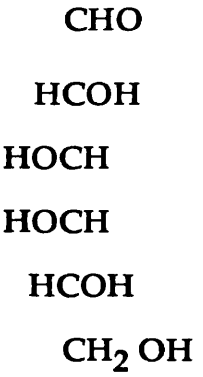


Fig. 9b: D-Galactose (Gal)

Fig. 9 (a & b) is showing 2 examples of the 7 simple mammalian sugars : Gal and the amino-sugar GalNAc.

The difference between Gal and GalNAc is an amino group (arrowed) which replaces the hydroxyl group at carbon atom 2 (Lehninger, 1970).

Polymers of two or more (up to 18) monosaccharides form complex chains with branches, called oligosacharides. Very long chains (polysacharides or glycans) can exist and may contain a single repeated monosacharide or up to 7 different monosaccharides.
The possible permutations conferred by such a system are enormous, with additional variations permitted by L or D forms and alpha or beta linkages to different carbons of sugar units. Complex carbohydrates or polysaccharides with a minor protein core are known as proteoglycans. Proteins with minor sugar are known as glycoproteins (or glycopeptides), but the distinction is not always clear.
Oligosaccharides linked to lipids (glycolipids) are major contituents of the cell membrane .

(8.2.) Carbohydrate detection in tissue culture and tissue sections

There is increasing evidence that the carbohydrate components of cell membrane molecules are important in defining cell types, and that changes in these components are associated with cellular differentiation, maturation and neoplastic transformation (Lis and Sharon, 1986B). Lectins therefore provide a range of readily available and well defined potential reagents for the identification of cell types (Sharon, 1988).

Lectin-binding carbohydrates appear to be conserved in many species, thus they appear of importance for the function to the organ in which they are present (Al-Alousi and Leathem,1989B)

At a tissue level, lectins have been used as specific probes for various cell types.

Mistletoe lectin I (MLI) and Ricinus communis lectin (RCA-I), both possessing D-galactose binding sites, selectively identified Microglial populations in aldehyde-fixed normal human and rodent brain tissues using paraffin and frozen sections (Suzuki et al, 1988).

In breast epithelium, peanut lectin (PNA), galactose binding lectin, has been reported to detect a marker able to discriminate between epithelial and myoepithelial lineage arising from rat mammary stem cells in culture and in tissue sections (Newman et al, 1979).

The cell membranes of essentially all mammalian cells contain complex glycoconjugates, which are integrated into it in the form of glycoproteins (bound to proteins) or glycolipids (bound to lipids). These glycoconjugates are important in cell-cell recognition and the particular value of lectins lies in their ability to distinguish the fine differences in the glycan component of cell glycoconjugates and to distinguish cell populations and their behaviour related to sugar expression (Hokomuri, 1989).

Using two lectins, Ulex Europeus lectin (UEA) I and lotus tetragonolobus lectin (LTA), with the same major sugar (fucose) specificity on the same tissues (human breast lesions), Walker (1984C) illustrated that these lectins have different binding affinities. Walker identified differences in behavioural characteristics between fucose-containing glycoconjugates having minor structural variations. This finding was illustrated by both single staining and double immunostaining methods using LTA-peroxidase and UEAI-alkaline phosphatase. In contrast to normal breast, in which there was consistent reactivity of all epithelium with LTA, a variable loss of binding was found within carcinomas. This bore no relationship to tumour differentiation; nor did it correlate with lymph node (LN)

metastasis. UEA had a variable reactivity with carcinomas, as it did with normal breast epithelium, and this had no correlation with differentiation. However, a relationship was found between the specific pattern of binding and LN status ($P < 0.02$).

Non-neoplastic epithelial cells bind lectin as well as tumour cells. However the distribution of lectin-binding carbohydrates is highly ordered in benign breast tissues so far examined but in neoplasms heterogeniety has been reported by many authors (Walker, 1983, 1984B, 1984C, 1984D; Al-Alousi and Leathem,1989C).

Franklin (1983) tested paraffin-embedded tissue sections of 20 breast lesions for the presence and distribution of lectin-binding carbohydrates (see table; 1, section 1.2.2.). The results were analyzed by fluorescence microscopy for the binding of wheat germ agglutinin (WGA), a GlcNAc and/or sialic acid binding lectin; Ricinus communis I (RCA I), a lactose binding lectin; Peanut agglutinin (PNA), a galactose binding protein; Soybean agglutinin (SBA), a galactose/GalNAc binding lectin; Dolichos biflorus agglutinin (DBA), a Gal-NAc binding lectin; Ulex europaeus agglutinin I (UEA I), a L-Fucose binding lectin, and Con A, a mannose binding lectin. Brightest and most consistent staining regardless of the nature of the breast lesion was obtained with WGA followed in approximate order of staining intensity by RCA, PNA, SBA/DBA and Con A. UEA-I stained many of benign breast lesions but no malignant lesions. Lectin binding carbohydrate in benign lesions was localized mainly along the apices of mammary epithelial cells but there was considerable variation in staining patterns among malignant tumours. The fluorescence microscopic arrangement of lectin binding carbohydrate appeared distinct for each malignant neoplasm of breast but was more consistent in benign conditions. Franklin concluded that the distribution of lectin-binding

carbohydrates is highly ordered in benign tissues examined and the disorganization of the carbohydrates in neoplasms may be exploited for diagnostic and therapeutic purposes.

Lectins have been used as probes for investigating differences in maturation and differentiation between normal and neoplastic cell membranes in human breast cancer tissues. The finding of Springer et al (1974) that cell membranes from human breast carcinomas absorbed the agglutinating principle from Peanut extracts more efficiently than cell membranes from normal or benign breast stimulated interest in the use of this lectin to study the differences in the carbohydrate expression between normal and malignant human tissues correlated with clinical behavior of these cancers (Newman et al,1979 ; Franklin, 1983; Walker, 1984D). There seem to be an agreement that PNA could possibly identify a marker of differentiation (Newman et al, 1979; Walker, 1984D).

Several lectins have been described to be marking the differentiation status of breast carcinomas e.g., Wheat germ lectin (WGA), which is a GlcNAc binding lectin; and Soybean lectin (SBA), which is a galactose/GalNAc binding lectin (Walker, 1984B, 1984D).

Walker (1983), applied fluorescent-labelled Concanavalin A (Con A), a lectin obtained from the Jack bean and has a high affinity for mannose and glucose group, to frozen and fixed processed sections of normal, hyperplastic and malignant human breast tissue. A difference in reaction was demonstrated between normal tissues (46 cases including 6 fibroadenomas) and carcinomas (60 cases), which was further accentuated when a lower concentration of ConA-FITC is used. Staining was consistently localised to the cell periphery in all benign tissue, in the well differentiated carcinomas and some of the

moderately differentiated tumours. A mixed pattern of reaction (staining of the cell periphery and cell cytoplasm) was seen in some tumours, whilst many of the poorly differentiated carcinomas showed cytoplasmic staining only. A significant correlation ($P<0.001$)was reported between the pattern of staining and the histological differentiation, with all the well differentiated carcinomas having peripheral staining and 10 out of 16 poorly differentiated tumours showing a cytoplasmic reaction. A significant correlation was also found between the degree of reactivity and differentiation ($P > 0.01$). There was no significant relationship ($P > 0.1$) between the pattern of staining and LN status (known on 22 carcinoma cases only). Walker concluded that Con A is strongly associated with tumour differentiation. However, Furmanski, Kirkland, Gargala et al (1981) have shown that the degree to which breast cancer cells react with Con A reflects the metastatic potential of these tumours but this was in a different system (cell suspension) and so is difficult to relate to tissue sections.

The lack of expression of GlcNAc as detected by lectins has been reported to be associated with metastasis.

Walker (1984B) studied the binding of peroxidase-labelled wheat germ agglutinin (WGA) to a series of 125 human breast carcinomas. The binding of WGA to breast carcinomas exhibited heterogeneity within neoplastic cells of the same tumour and between carcinomas. In relation to cell reactivity, the heterogeneity was significantly correlated to tumour differentiation ($P<0.001$), with fewer cells reacting in those carcinomas that showed loss of histological differentiation. A significant association ($P < 0.01$) was found between decreased reactivity and the presence of axillary LN metastasis, but a lack of correlation between virtual absence of reactivity and nodal metastasis was reported.

Al-Alousi and Leathem (1989C) investigated the presence of Pokeweed (Phytolacca americana) lectin receptors on formalin-fixed paraffin-embedded sections of primary breast cancers, using both indirect immunoperoxidase and avidin-biotin staining method on 100 cases followed for up to 10 years. The Pokeweed lectin (PWA) used is a mitogen which has an affinity for GlcNAc. PWA receptors were identified in 43% of the primary breast cancer cases examined irrespective of the patient blood group. Benign cells gave only luminal positivity, In cancer cells, the staining was either cytoplasmic, or at the cell surface. A significant correlation ($P<0.014$) was found between absence of reactivity and LN involvement. When the cases were divided according to age into two age groups: over and under 50 years of age, the correlation was stronger in the over 50s (including 50-yr-olds) ($P<0.012$), while in younger patients there was no significant correlation ($P<0.390$). No significant correlation was found with tumour size, histological type or with over all survival. However, the correlation with disease free interval did suggest that the cases that did not stain had a recurrence rate earlier on than those that stained.

The expression of GalNAc as detected by Helix pomatia lectin (HPA) was reported to be associated with local axillary LN metastasis (Leathem et al, 1984; Leathem et al, 1985) and locoregional recurrence and short survival (Fenlon et al, 1986; Fenlon et al, 1987).

Leathem and Brooks (1987) correlated the binding of HPA on formalin-fixed paraffin-embedded tissue sections of 179 patients with primary breast cancer followed-up for 15-20 years, with the clinical behaviour of the disease. The patients were divided into two age groups: premenopausal (50 years olds and less than 50) group and post

menopausal group (those over 50 years). In premenopausal patients, there were significant differences between the groups with and without HPA staining in time to first recurrence ($P<0.01$) and in survival time ($P< 0.001$). These differences persisted over 15 years. However in postmenopausal patients, there was no significant difference in time to first recurrence or survival time between the groups with and without HPA staining.

(8.2.) Carbohydrate detection in fine needle aspirations (FNAs)

Lectins may be useful tools if applied on tissues removed at operation as potential markers of clinical behaviour in breast cancer. Even more useful could be the demonstration of lectin receptors in cytological smears of breast carcinoma from fine needle aspirations (FNAs), which could be taken in Out-Patient clinics, to assist in early management decisions.

Henry et al (1987) investigated PNA binding patterns in fine-needle aspirations (FNAs) of breast lesions to evaluate the possible correlation between PNA binding patterns and histopathologic assessment of malignancy. A PNA immunoperoxidase method was applied to smears from a group of 15 benign and malignant breast lesion. Cytostructural localization of PNA binding was seen in both benign and malignant breast lesions. Histopathological sections showed a similar staining pattern. Henry et al concluded that PNA staining patterns appear to be of little diagnostic utility in the assessment of malignancy.

Demonstrating lectin receptors in FNAs was also attempted by Al-

Alousi and Leathem (1989D), in a pilot study, using as a model HPA binding, in order to find out if, by using lectins of prognostic predictive value, FNAs could then aid to speed up the staging service. The material used consisted of 27 FNAs that were performed in the breast clinic by breast surgeons. Aspirates were normally scanty, of poor technical quality and averaged 3 smears per case. Two were sent for routine staining for diagnosis and the remaining slide for HPA staining. The corresponding paraffin-embedded tissues were subsequently taken for comparison. The majority (17) of the aspirates contained too few cells to be suitable for immunocytochemical staining. Only 10 cases were found to be suitable. Of these cases, 7 showed positivity. Theseresults corresponded to subsequent histology both in terms of positive or negative staining by HPA and in the cytological localization of the positive staining. Al-Alousi and Leathem concluded that the immunocytochemical staining with lectins could be applied succesfuly to FNAs. This could provide a simple, speedy and inexpensive staging service but the usefulness is totally dependent on adequate FNAs.

Aims

This work was carried out in order to study the carbohydrate expression on primary breast cancer cells detected by mistletoe lectins and to assess their role in clinical behaviour of patients with primary breast cancer.

These aims to be achieved by:

1- Obtaining pure preparations of the mistletoe lectins.

2- Exploring the underlying relationship between mistletoe lectins and cancer cells, by seeking binding of lectins to paraffin sections of primary breast cancers, using immunohistochemical methods.

3- Correlation of clinical behaviour of primary breast cancer patients (followed for up to 11 years) with the binding of mistletoe lectins to their paraffin sections, comparing histological type and size of primary, blood group, lymph node status, disease free interval (recurrence) and survival of patients.

2. MATERIALS , METHODS AND RESULTS

FOR ISOLATING LECTINS

2.1. Isolation of European Mistletoe (*Viscum album*) lectins No. 1

European Mistletoe plant (*Viscum album*) grown on apple tree, imported from Britanny/France, was purchased from Chivers flower shop in Charlotte Street, London W1, fresh in mid December and was used to isolate Mistletoe lectins in this experiment. The isolation procedure was briefly as follows:

2.1.1. Making a crude extract of Mistletoe whole plant (leaves, stems and berries).

2.1.2. Ammonium sulphate $(NH_4)_2SO_4$ precipitation of the proteins from Mistletoe crude extract.

2.1.3. Gel chromatography of the precipitated proteins, using Bio-Gel P-200.

2.1.4. Ion-exchange chromatography for the material that was eluted from the Bio-Gel column and that was shown to contain lectin by a haemagglutination test. The anion exchanger used was DE52.

2.1.5. Repeat ion-exchange chromatography, using DE52, with shallower elution gradient, for the material that was eluted from the DE52 column (step No. 2.1.4) and that was shown to contain lectin by a haemagglutination test.

2.1.6. Affinity chromatography of the material eluted from the DE52 column (step No. 2.1.5.) and that was shown to contain lectin by a haemagglutination test.

2.1.1. Making an extract of whole Mistletoe plant

2.1.1.1. Extraction of Mistletoe lectin(s)

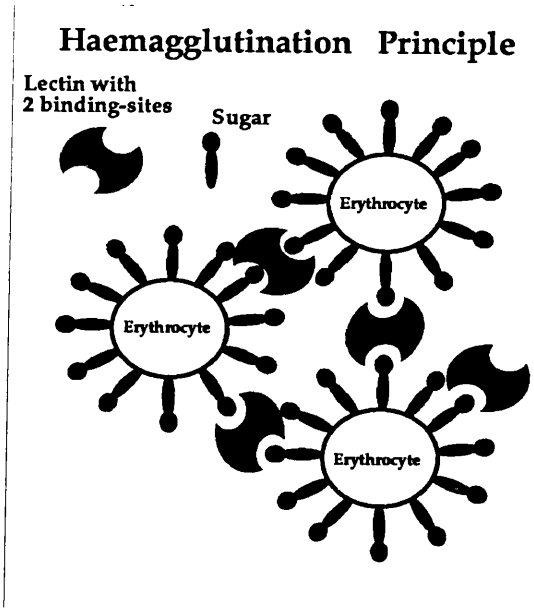
Leaves, stems and berries of European Mistletoe (*Viscum album*) (100g) were minced and then stirred with 1 litre of 0.5M NaCl solution containing 0.1M galactose, overnight at 4°C. The galactose was included to block binding of lectins to carbohydrate components in the homogenate. The mixture was filtered and then centrifuged at 10,000g for 10 minutes. The pellet was discarded.

2.1.1.2. Testing the extract

The supernatant was tested for haemagglutination using fresh red blood cells (RBCs) of blood group B to titre the lectin.

Principle of haemagglutination

The haemagglutination test is based on the principle that a multivalent lectin will bind to and cross-link carbohydrate on RBCs. Since most lectins have more than one binding site, more than one RBC may bind to one molecule of lectin, forming a cross-linked or agglutinated cluster of cells (as shown below in Fig. 10).



Haemagglutination was carried out as follows:

Fresh human RBCs blood group B were washed twice in phosphate buffered saline (PBS) pH 7.2 (appendix; 1) and diluted to 1%. To each well of 96-well microtitre plates , 10µl of serial dilutions (1:2), up to dilution of 1:256, of the supernatant of Mistletoe crude extract were placed. 10µl of the RBCs suspension were added to each well and after mixing the plate was stored at room temperature for a maximum time of 30 minutes to allow the erythrocytes to sediment. In the absence of agglutination, the RBCs formed a small button in the centre of the well, whereas agglutinated cells were deposited as a film covering the whole buttom of the well. The lowest concentration of added protein giving visible agglutination was determined.

2.1.1.3. Results of extraction

Haemagglutination revealed that the supernatant would agglutinate RBCs down to a dilution of 1:16. The supernatant of the crude extract was subjected to the following procedures.

2.1.2. Ammonium sulphate $(NH_4)_2SO_4$ precipitation (Hudson and Hay, 1976)

2.1.2.1. Principle of ammonium sulphate precipitation

The principle behind this technique is that as the salt concentration of the medium is raised there is an interference between the interaction of water molecules with the charged polar groups on protein molecules, thus rendering them less hydrophilic. This allows a greater hydrophobic interaction between protein molecules and so they progressively become insoluble.

2.1.2.2. Preparation of saturated ammonium sulphate

First a saturated ammonium sulphate solution was prepared (appendix; 2) and an experiment was carried out to find out the best ratio of the crude extract to the ammonium sulphate solution for precipitating lectins (as in appendix; 3).

2.1.2.3. Precipitating lectin(s) from Mistletoe extract

Ratio of 7 parts extract : 3 parts ammonium sulphate was found to be the best ratio to precipitate the lectin(s) (by haemagglutination). So 700 mls of crude Mistletoe extract was mixed with 300 mls of saturated ammonium sulphate solution. The mixture was allowed to settle overnight at $4^{o}C$ and the precipitate collected by centrifugation at 10,000 for 10 minutes, dialysed overnight against distilled water **DW** and then resuspended in a minimum volume of 0.5 M NaCl.

The collected proteins were subjected to the following procedures:

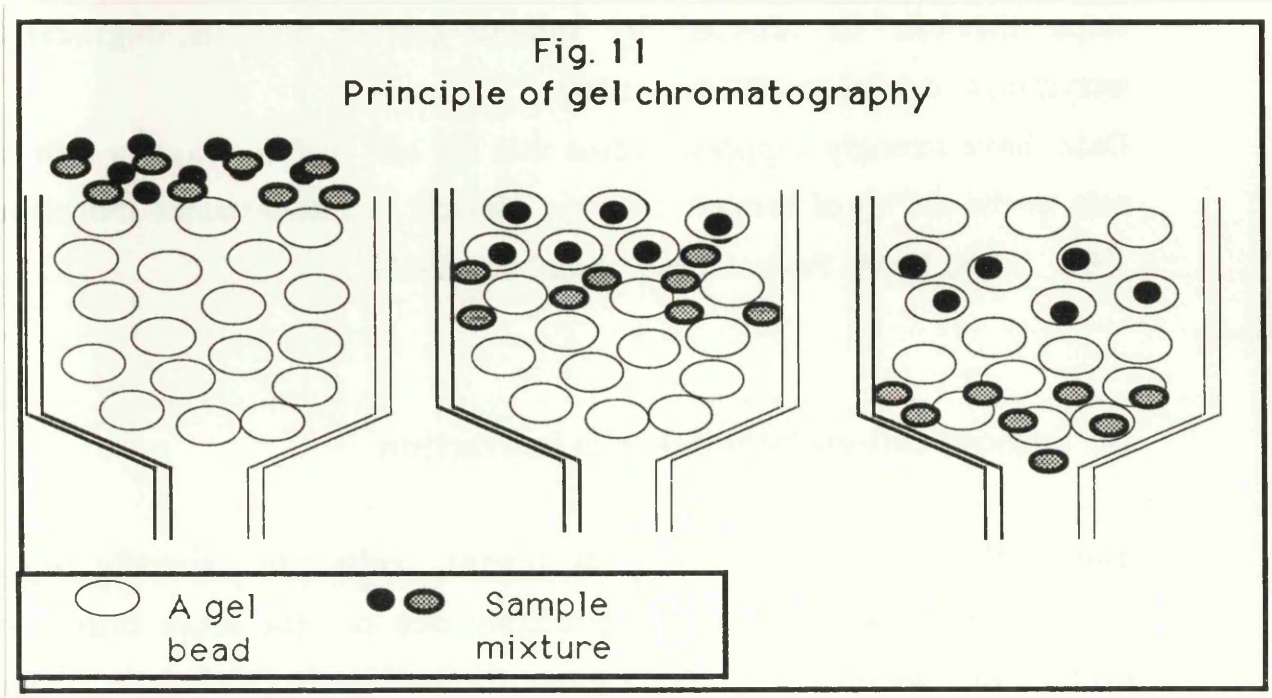
2.1.3. Gel exclusion chromatography (Porath and Flodin, 1959)

2.1.3.1. Principle of gel chromatography

Also called gel filtration, is a liquid chromatographic method which separates molecules primarily according to differences in their molecular weight (as shown below in Fig. 11). (details on mechanism of action are found in appendix; 4) .

This separation, based on differences in molecular size, can be influenced by other phenomena such as ion exchange on the same matrix or even interaction between carbohydrates of gel matrix and lectins. Bio-Gel P-200 is a porous polyacrylamide bead which carries no charge or carbohydrate content, exhibiting molecular weight exclusion limits from

30.000-200.000D (Bio-Rad) and was used for this purpose.



2.1.3.2. **Separation of Mistletoe lectin(s) by gel chromatography**

The material obtained from the ammonium sulphate $(NH_4)_2SO_4$ precipitation was applied to the Bio-Gel P-200 column (see appendix, 4).

The conditions for Bio-Gel P200 column chromatography in a typical run were as follows:

The diameter of the column = 2.6cm

The height of the gel bed = 80 cm

The volume of the column = 80 X (1.3)2X 22/7 = 282 cm^3 of gel

The volume of sample = 10 mls.

Flow rate = 15 mls/hr

Fraction size = 2 mls.

Void volume = 95 mls.

Eluent = Tris buffered saline (TBS) pH=7.2 (appendix; 5)

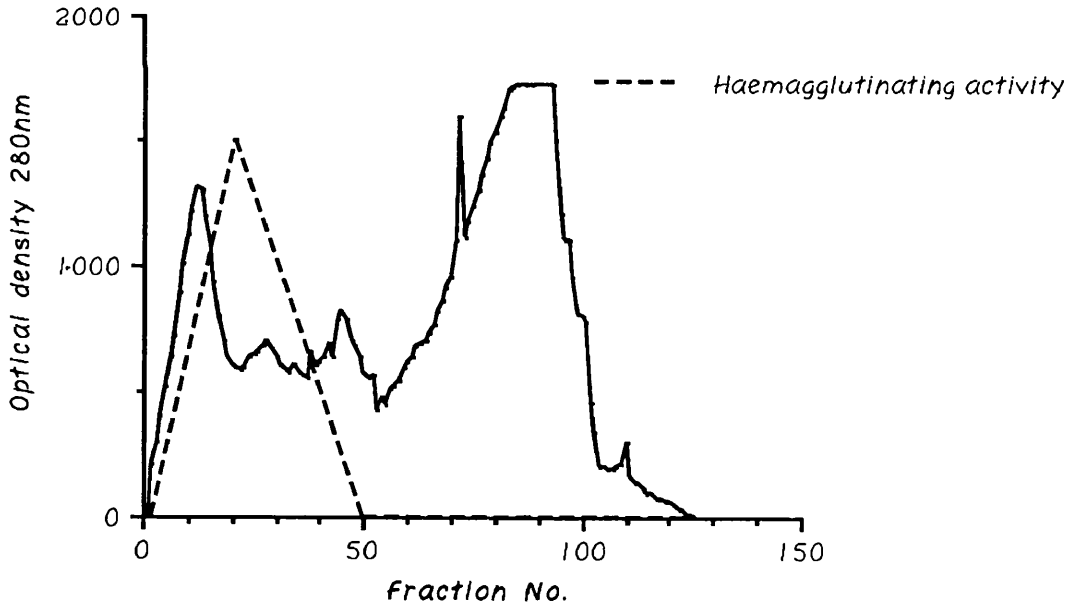
Each fraction from the column was tested for protein concentration by absorption using a Shimadzu spectrophotometer wavelength **WV**=

280nm and was tested by haemagglutination for the presence of lectin.

2.1.3.3. Results of P200 separation

Gel chromatography revealed the presence of many components (many peaks) (Fig. 12). When these peaks were tested for the presence of lectin by haemagglutination test, it was found that many peaks contained lectin (see Fig. 12). However, the haemagglutinating activity suggested the presence of one lectin peak. The fractions of the peaks that showed haemagglutinating activity were mixed together and were subjected to the following procedures.

Fig. 12 Gel filtration (Bio-Gel P-200 column) of mistletoe crude extract



2.1.4. Ion-Exchange Chromatography

2.1.4.1. Principle of ion-exchange chromatography

Ion-exchange chromatography separates proteins by taking advantages of net charge differences (as shown below in Fig. 13). Most ion- exchange experiments are performed in two main stages. These are:

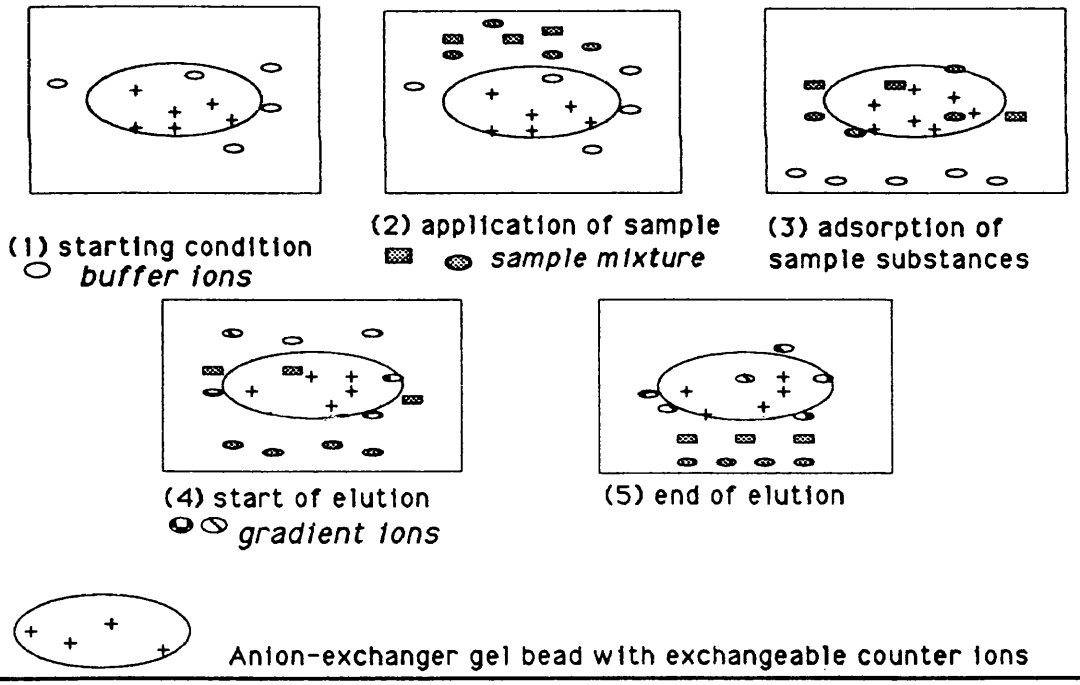
(1) the addition and binding of substances to the ion exchanger.

(2) the removal of these substances one at a time, separated from each other.

Fig. 13

Principle of Ion Exchange Chromatography

(modified from Pharmacia (1987))



Elution may be affected either by changing the pH of buffer passing through the column, thus affecting the charge on the protein molecules, or by increasing the molarity of the buffer, thus providing more salt ions to compete with the proteins for charged groups on the exchanger. Separation is possible since substances normally have different electrical

properties. The exchanger such as diethylaminoethyl, DEAE (which is an anion exchanger used for adsorption of negatively charged molecules), and carboxymethyl, CM (which is a cation exchanger used for adsorption of positively charged molecules) determine the functional characteristics of the adsorbent (Fahey and Terry, 1973). DEAE was used in this study with elution by increasing salt (NaCl) molarity.

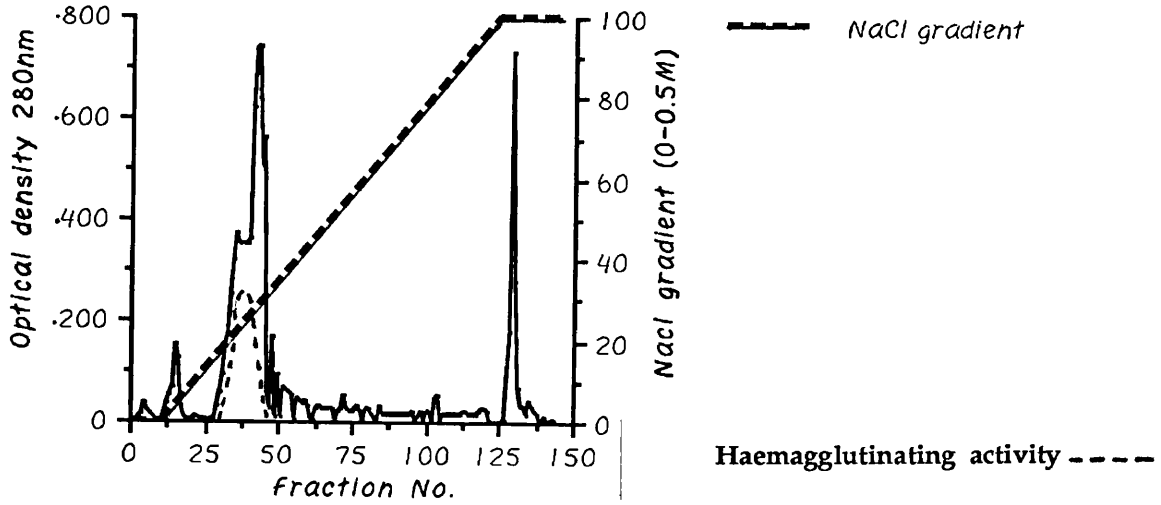
2.1.4.2. Separation of Mistletoe lectin(s) by ion-exchange chromatography

The pooled fractions from the Bio-Gel P-200 column which were haemagglutination test positive, were dialysed against distilled water then put on ion- exchange resin DE-52 (see Appendix 6 for details). Eluting the different components was achieved using a gradient of 0-0.5 M NaCl . The protein concentration in each fraction was measured using a spectrophotometer to measure absorption at WV 280nm. Each fraction from the column was tested by haemagglutination for the presence of lectin.

2.1.4.3. Results of anion exchange chromatography

The elution profile consisted of many peaks (many components) (Fig. 14). When these peaks were tested for the presence of lectin by haemagglutination , again it was found that many peaks contained lectin. However, haemagglutinating activity suggested the presence of one peak of component with haemagglutinating activity (see Fig. 14). The fractions containing the peaks that were haemagglutination positive, were mixed and subjected to the following procedures.

Fig. 14 Ion-exchange Chromatography (DE-52 column) of the haemagglutination positive material from the P-200 column



2.1.5. Repeated anion exchange chromatography

2.1.5.1. Separation of Mistletoe lectin(s) by 2nd ion-exchange column

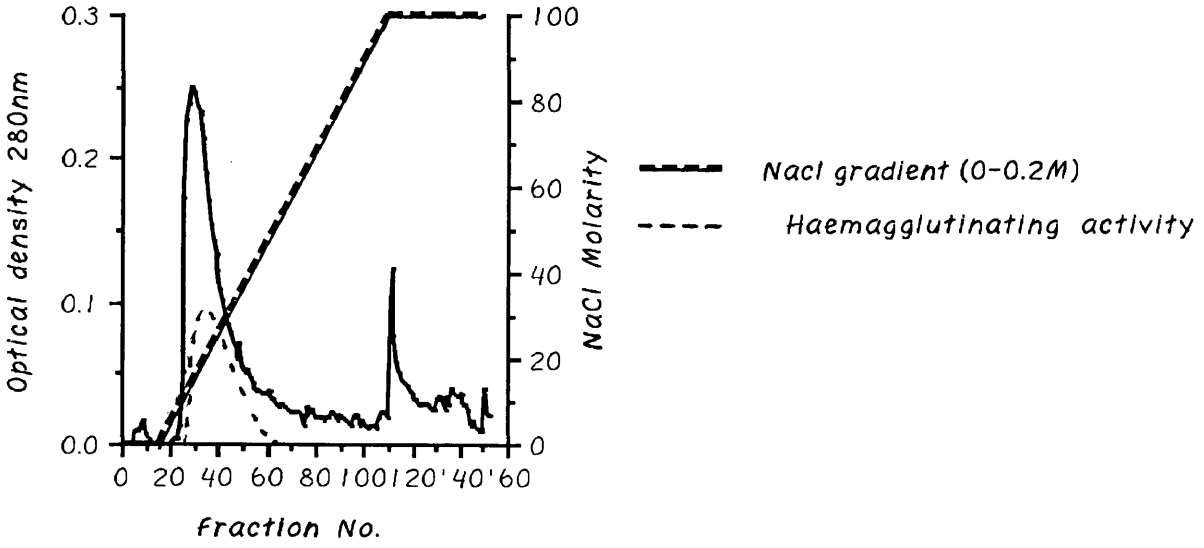
The pooled fractions from the DE-52 column which were haemagglutination positive were dialysed against distilled water and put on a second anion-exchange column (DE-52). Eluting the different components was achieved using a shallower gradient of NaCl molarity (0-0.2 M NaCl). Each fraction from the column was tested for protein concentration by absorption at wavelength WV 280nm and by haemagglutination for the presence of lectin.

2.1.5.2. Results of second anion exchange chromatography

The second anion exchange chromatography revealed the presence of fewer peaks (Fig. 15). When these peaks were tested for the presence of lectin by haemagglutination, it was found that the main peak in the elution profile contain lectin. The fractions of the lectin peak were

mixed and subjected to the following procedures.

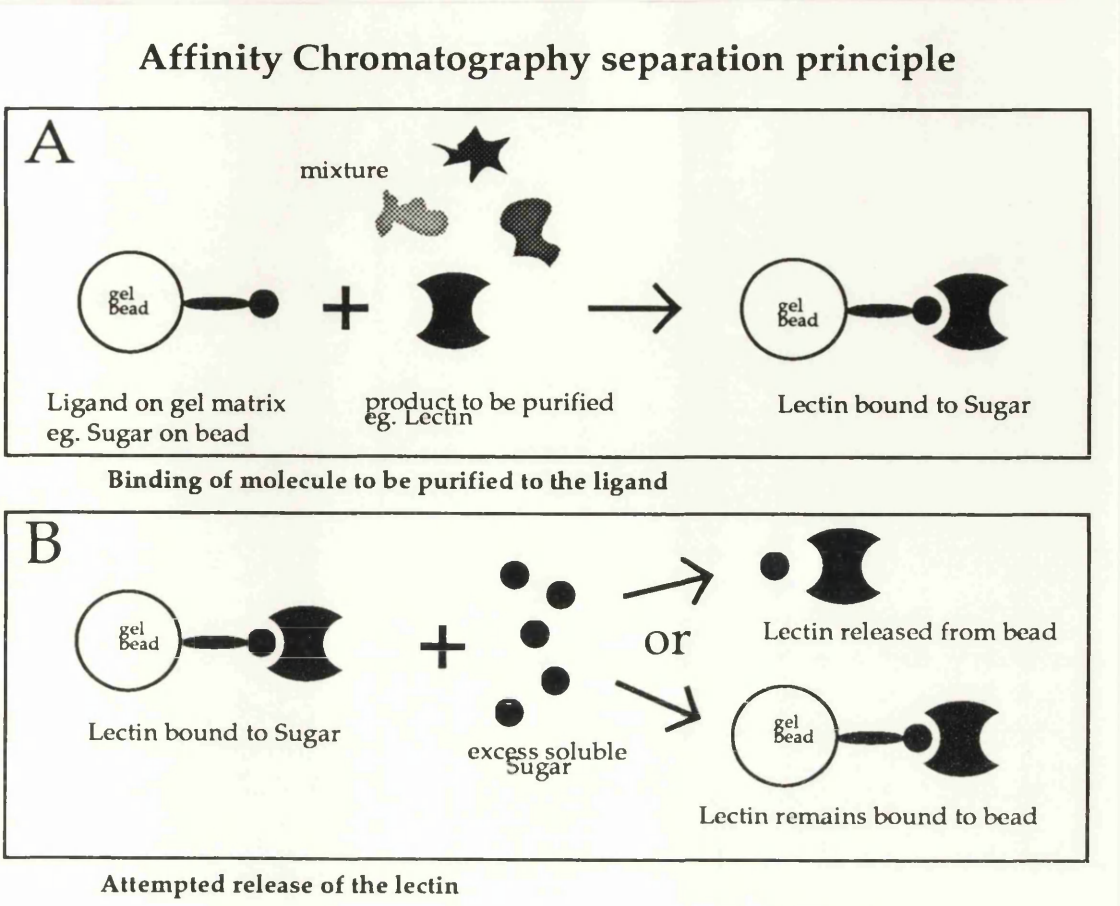
Fig. 15 Second Ion-exchange chromatography (DE-52 column) of the haemagglutination positive material of the 1st DE-52 column



2.1.6. Batch affinity chromatography

2.1.6.1. Principle of affinity chromatography

Affinity chromatography depends, in principle, upon the specific and reversible attachment of e.g. protein to a binding substance (called a ligand) which has itself been covalently attached to an insoluble polymer or gel matrix. A carbohydrate ligand with which the lectin interacts is insolubilized, the lectin is adsorbed as the extract is percolated over the adsorbent, and displacement of bound lectin is accomplished by elution, either with a sugar that competes for lectin sites with the specific adsorbent or by altering the nature of the eluent (eg. by lowering the pH, or by increasing ionic strength) (Goldstein and Poretz, 1986) (as shown below in Fig. 16).



2.1.6.2. Separation of Mistletoe lectin by affinity chromatography

The pooled fractions from the 2nd DE-52 column which were haemagglutination positive, were applied to a column of Sepharose 4B beads covalently coupled to lactose (Sigma). Lactose was used because the Mistletoe lectin is known to have an affinity for this (Franz, 1986). The material was incubated with the lactose-sepharose beads at room temperature on a shaker. the binding of the lectin to the beads was monitored by haemagglutination, using the supernatant, every two hours. When the haemagglutination test of the supernatant was negative, the unbound material were then passed and the column was washed with TBS until the washings became protein free (absorption reading at 280nm by spectrophotometer = 0). The lectin was then eluted with 0.2M galactose by incubation overnight on a shaker. The eluted material was dialysed overnight against TBS, to remove the

galactose, tested for protein concentration by spectrophotometer absorption reading at WV = 280nm and was tested by haemagglutination for the presence of lectin.

2.1.6.3. Results of affinity chromatography

The eluted material was subjected to haemagglutination test which revealed that the lectin present in the eluate would agglutinate human RBCs (blood group B) up to dilution of 1:128.

2.1.6.4. Problems of low yield using affinity chromatography

Unfortunately, a small yield of the purified lectin was obtained (only 7mg out of 100g plant).

Since we found that we could only obtain a small yield of the purified lectin , we therefore had to determine whether: (i) the lectin had bound to the beads; (ii) if it could have been lost in the washing procedure; (iii) whether it had been eluted or whether (iv) it remained on the beads even after elution.

To determine this, a simple test was developed (Al-Alousi and Leathem, 1989E). This test allows the detection of the presence of Mistletoe lectin on beads by looking for microscopical haemagglutination, or binding, of RBCs onto sepharose 4B beads covalently coupled to lactose, for which Mistletoe lectin has an affinity, in a series of tests corresponding to the different stages of affinity chromatography.

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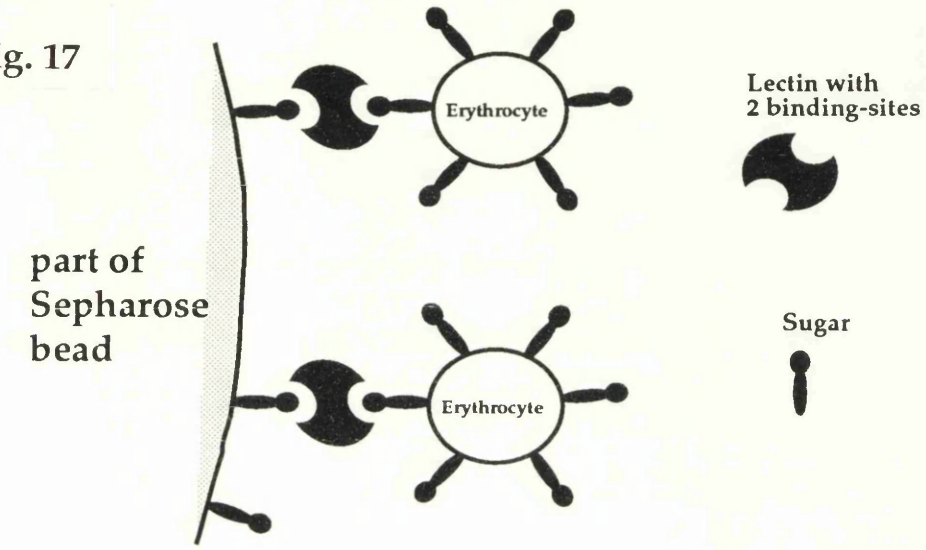
2.1.6.4.1. A simple test for monitoring the binding of lectins in affinity chromatography.

The test is based upon Mistletoe lectin binding to beads but also to erythrocytes (as shown below in Fig. 17).

Fig. 17



Erythrocytes linked to Sepharose bead by Lectin

Haemagglutination activity was determined in a 1% suspension of washed human erythrocytes of blood group B. To each 30 µl of human RBCs, 30 µl of 1% suspension of Sepharose 4B coupled lactose were added, on a haemagglutination plate. The mixture was left for 5 minutes at room temperature then a drop of the mixture was put on a glass slide and viewed under a phase contrast microscope. This test was performed using beads at different stages in the affinity chromatography procedure:

1- Haemagglutination test using the beads after application of the material that was obtained from the second ion-exchange column and

after washing with TBS.

2- Haemagglutination test after eluting with 0.2M, 0.5M and 1M of the corresponding sugar and washing with TBS.

3- Haemagglutination test after eluting with HCL/Glycin buffer pH 2.6 (appendix 7) and washing with TBS.

4- Haemagglutination test on beads bearing the corresponding sugar washed with TBS was used as negative control.

2.1.6.4.2. Results of simple test

In the negative control, the RBCs were seen scattered evenly among the gel beads on the slide which indicated that there was no lectin in the mixture (Fig. 18), whereas in the tests carried out on beads after incubation with material that contained the lectin, the RBCs were seen binding together on and around the beads but not away from them which indicated that the lectin had bound to the beads (Fig. 19 & 20).
The results showed that some Mistletoe lectin remained bound to the beads after elution with a corresponding sugar of a high molarity, and even after elution with acid buffer, indicating that it is perhaps not possible to elute all Mistletoe lectin by affinity chromatography under these conditions.

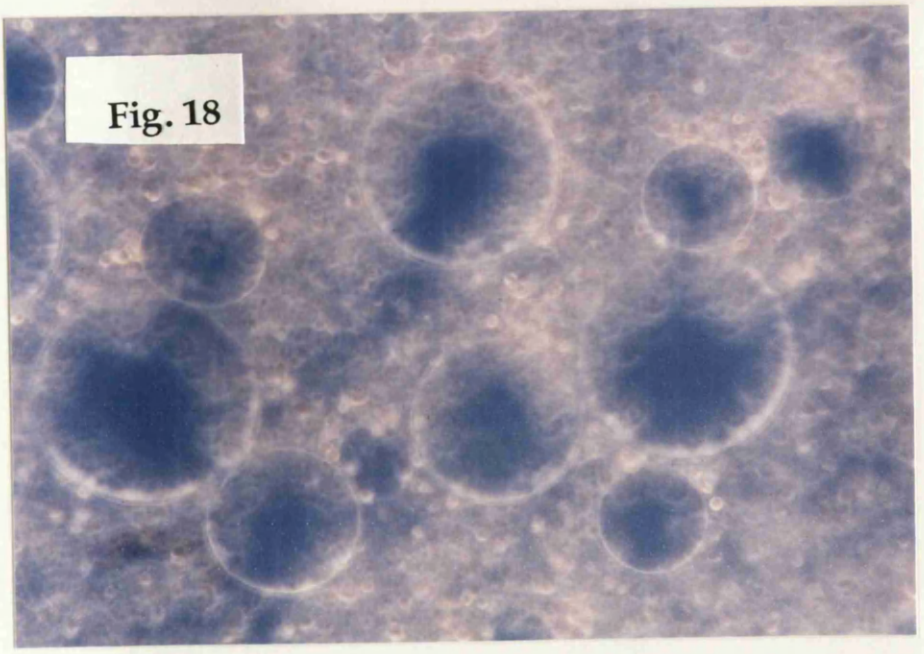


Fig. 18 Negative control

No agglutination is seen on the beads. X 40

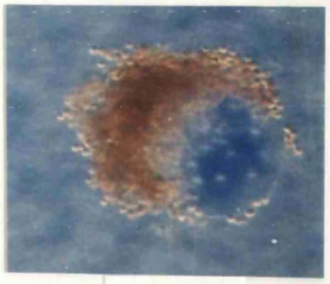


Fig. 19

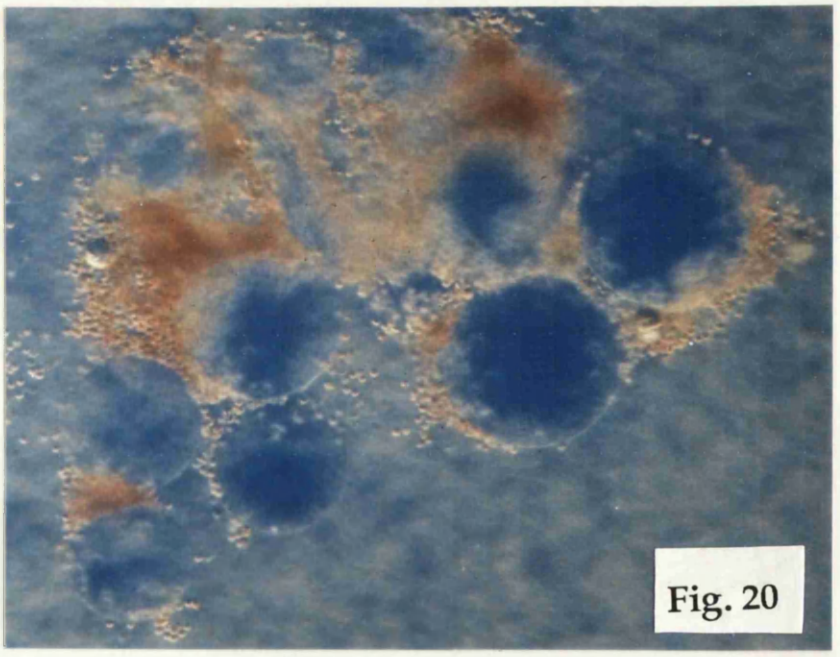


Fig. 19 One bead with agglutination of RBCs around it. X 40

Fig. 20 A cluster of beads carrying agglutinated RBCs. X 40

2.2. Identification and characterization of the purified lectin

The eluted material from the Sepharose-lactose column which was shown to contain lectin by haemagglutination test was also tested by (i) high performance (pressure) liquid chromatography (HPLC)/gel chromatography to ascertain the purity of the lectin, (ii) Polyacrylamide gel electrophoresis to find out the purity of the lectin and its molecular weight, and (iii) haemagglutination inhibition test to find out its carbohydrate specificity or affinities.

2.2.1. High performance (pressure) liquid chromatography (HPLC)

(Wilson and Goulding, 1988).

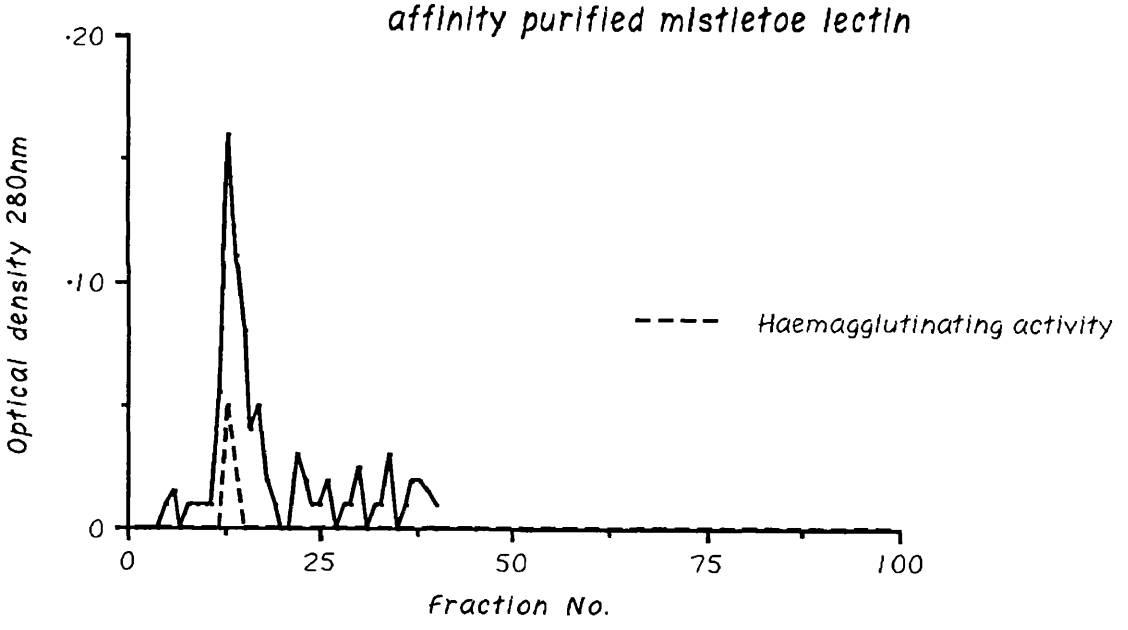
All of the forms of column chromatography so far mentioned rely on gravity or low pressure pumping systems for the supply of eluant to the column. The consequence of this is that the flow rates achieved are relatively low and this gives greater time for band broadening by simple diffusion phenomena. The use of faster flow rates is not possible because it creates a back-pressure which is sufficient to damage the matrix structure of the stationary phase, thereby actually reducing eluent flow and impairing resolution. Recent development in column chromatography technology has resulted in the availability of HPLC, in which smaller particle size stationary phases can withstand pressures and pumping systems which can give reliable flow rates are used. These developments have resulted in faster and better resolution. The columns used for HPLC are generally made of stainless steel and are manufactured so that they can withstand high pressures. The stationary phase for exclusion separation are generally porous silica glass, polysterene or polyvinylacetate beads. In this study a prepacked

column of TSK G-3000SW, which is a hydrophilic silica gel was used. the particle size of the gel is 10 μm that allow protein separation in normal saline of a MW range of 1,000-300,000.

2.2.1.1. Results of HPLC/Gel chromatography

Gel chromatography using HPLC revealed a main peak, the fractions of which possessed haemagglutinating activity (Fig. 21). These fractions were pooled and used in this study.

Fig. 21 HPLC/ Gel Chromatography of the affinity purified mistletoe lectin



Using HPLC was under the supervision of my colleague Dr. A. K. Sesay (Histopathology Dept./UCMSM)

2.2.2. Polyacrylamide gel electrophoresis (PAGE) Laemmelie 1970)

2.2.2.1. Principle of PAGE

Proteins migrate in an electric field. This migration is dependent on the charge, size and shape of molecules. However, in the presence of Sodium Dodecyl Sulphate (SDS) proteins bind the SDS and all become negatively charged. When these SDS coated proteins are placed in an electric field, the separation of the proteins will depend only on their size and shape. By varying the concentration of the polyacrylamide gel, used as support medium for the electrophoresis, different molecular weight ranges (appendix 8A) may be separated. Proteins may be fractionated in their native state, but better resolution is usually obtained if the disulphide bonds are first reduced, allowing separation of the individual peptide chain. After heating to 100^{o}C in the presence of reducing agents (mercaptoethanol) and SDS, proteins unfold and bind about 1.4g SDS per gram protein. The stacking gel is useful in that it leads to sharp, straight bands and so allows the loading of variable volumes of sample (more about the principle behind the stacking gel effect is in appendix 8B).

2.2.2.6. Running the gel

The samples were prepared (appendix 8E). The running gels (7.5% SDS-PAGE under non-reduced conditions and 12% SDS-PAGE under reduced condition) and the stacking gels were prepared (appendix 8C and 8D) and let to set, then the gel apparatus was connected to the power supply and the gel was run (appendix 8G) at constant voltage supply.

Molecular weight standards (Sigma) (appendix 8F) were included in each run. Crude Mistletoe extract was also run by PAGE for comparison. After running , the gel was stained by Coomassie blue (appendix 8H).

2.2.2.9. Results of PAGE

Using 7.5% SDS-PAGE under non-reduced conditions, the lectin gave one large strong band (2 bands very close to each other?) in the zone of molecular weight MW of 60KD and in addition 2 more fainter bands were seen in the zone of a MW of 34kD and 29KD (Fig. 22).
Using 12% SDS-PAGE under reduced condition (using mercaptoethanol), the lectin gave rise to 2 major bands. The heaviest band was of a MW of 34kD. The lightest band was of a MW of 29KD. The two bands appeared to consist of 2 further bands. In addition, a very faint band was seen corresponding to MW of 60KD (Fig. 23).

Using 12% SDS/PAGE under reduced condition, the crude extract was shown to contain many components (Fig. 24).

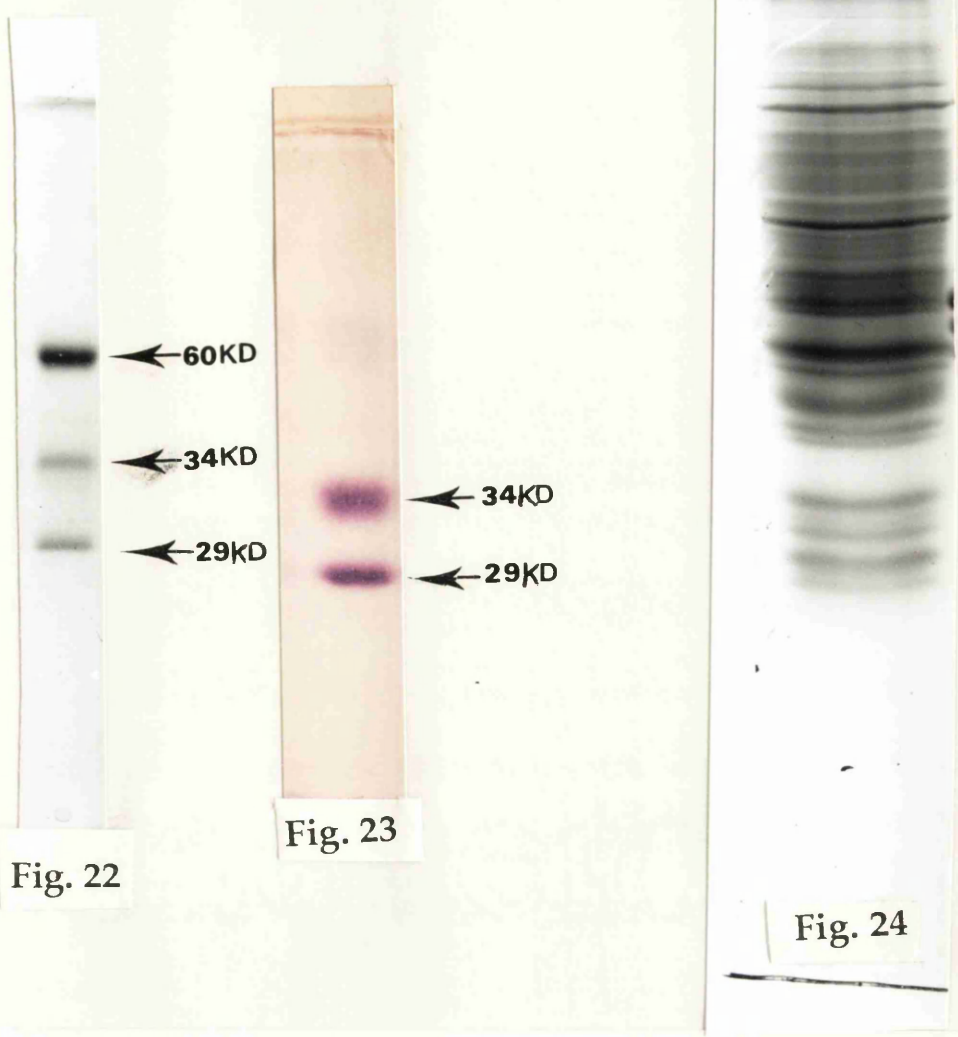


Fig. 22

Fig. 23

Fig. 24

Fig. 22
SDS/PAGE (7.5% gel stained with Coomassie blue, under non-reduced conditions).
The isolated, affinity purified mistletoe lectin, showing a major band in the zone of MW of 60KD. In addition, 2 more faint bands are seen corresponding to MW of 34KD and 29KD.

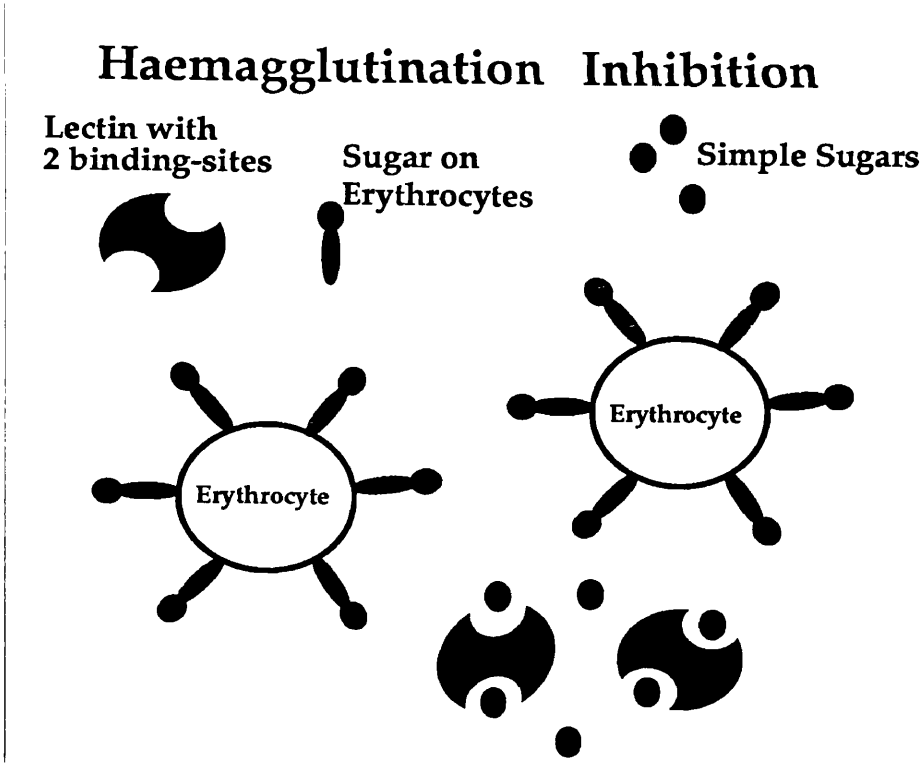
Fig. 23
SDS/PAGE (12% gel stained with Coomassie blue, under reduced conditions).
The affinity purified lectin showing 2 major bands corresponding to MW of 34KD and 29KD. A very faint large band is seen in the zone of MW of 60KD.

Fig. 24
SDS/PAGE (12% gel stained with Coomassie blue, under reduced conditions).
Crude mistletoe extract containing many components, of which a band of MW of 120KD, a band of MW of 60KD, a band of MW of 34KD and a band of MW of 29KD.

2.2.3. Haemagglutination inhibition test

2.2.3.1. Principle of haemagglutination inhibition

If the binding sites of the lectin in a suspension are already bound to carbohydrates the lectin will not be able to bind to carbohydrates on RBCs so that when RBCs added to a solution contains lectin and a carbohydrate that the lectin could bind to i.e. has an affinity to, these RBCs will not be agglutinated. This is the principle behind the haemagglutination inhibition test (as shown below in Fig. 25). This is why this test was used to find out simple sugar-specificity of lectins.



2.2.3.2. Method

Haemagglutination inhibition tests were performed as follows:
to 10 µl of a 2-fold serial dilution of each simple sugar tested (D-galactose, glucose, N-Acetyl- galactosamine, N-Acetyl Glucosamine, mannose, and fucose), 10µl of lectin solution with haemagglutinating activity was added. After incubation at 37°C for 1 hr, 20µl of 1% of fresh washed human RBCs of blood group B was added. After 1 hr incubation at 37°C, the degree of agglutination was estimated. Negative control was by using PBS instead of lectin solution. So the RBCs was incubated with the simple sugar and PBS.

2.2.3.3. Results of haemagglutination inhibition test

The results show that the lectin was strongly inhibited by D-Galactose (down to 0.001M of the sugar). The lectin was weakly inhibited by N-Acetyl galactosamine (GalNAc) (down to 0.2M of the sugar).

As the most significant work to date on Mistletoe has been carried out by Prof. H. Franz in East Berlin/German Democratic Republic (GDR), where he worked on Mistletoe from different sources, a collaboration was arranged for myself (Maha Al-Alousi) to attend the Staatlich Institut fur Immunpraparate und Nahrmedian in East Berlin/GDR and work with Prof. H. Franz and his co-workers in May/June 1989, when the following experiment was carried out.

2.3. Isolation of European Mistletoe (*Viscum album*) lectins No.2

Three lectins were isolated from crushed and powdered plant material from Mistletoe grown on the Locust tree (*Robinia pseudoacacia*) using the following procedure:

2.3.1. Making a crude extract of Mistletoe

Dry Mistletoe plant grown on the Locust tree was crushed and 1 part of the Mistletoe powder was mixed with 5 parts of distilled water. The mixture was left for 3 hrs with occasional stirring.

2.3.2. A batch cation exchange chromatography

2.3.2.1. The supernatant of the extract was taken, checked for the presence of lectins by haemagglutination test, using RBCs blood group B, and its pH was adjusted to 4.0.

2.3.2.2. To each 1 litre supernatant, 2.5g of SP-Sephadex C-50 (Cation exchanger beads) was added, left for 30 minutes with stirring.

2.3.2.3. After making sure that all the lectin has bound to the beads, by testing the supernatant by haemagglutination using RBCs blood group B, the supernatant was decanted.

2.3.2.4. The SP-Sephadex C-50 beads were washed with Sodium-acetate buffer (0.1M acetic acid in distilled water corrected to pH 4.0 with sodium hydroxide) until the washing was protein free (reading by absorption at WV 280nm using a spectrophotometer = 0).

2.3.2.5. The beads then were packed into a column and the bound material to the beads was eluted with 0.5M Nacl with the range of pH 7.5 - 7.8. The eluted material was tested for the presence of lectins by haemagglutination using RBCs blood group B.

2.3.3 Ammonium sulphate $(NH_4)_2SO_4$ precipitation

To each 10 mls of the solution of the eluted material from the SP-Sephdex C-50, 6g of solid ammonium sulphate was added. The mixture was left overnight at 4^oC. The precipitated material was collected by centrifugation and the supernatant was discarded.

2.3.4. Affinity chromatography

The proteins obtained from the ammonium sulphate precipitation were diluted 1:8 in distilled water, checked for the presence of lectins by haemagglutination using blood group B, and applied onto a column of galactosyl-Sepharose. The effluent (the material that did not bind to the beads and was passed) was kept. The bound material was eluted from the column by 0.2M galactose in 0.9% Nacl. the material eluted was subjected to haemagglutination , which revealed that the material possessed haemagglutinating activity. This is Franz Mistletoe lectin No. 1 "MLI").

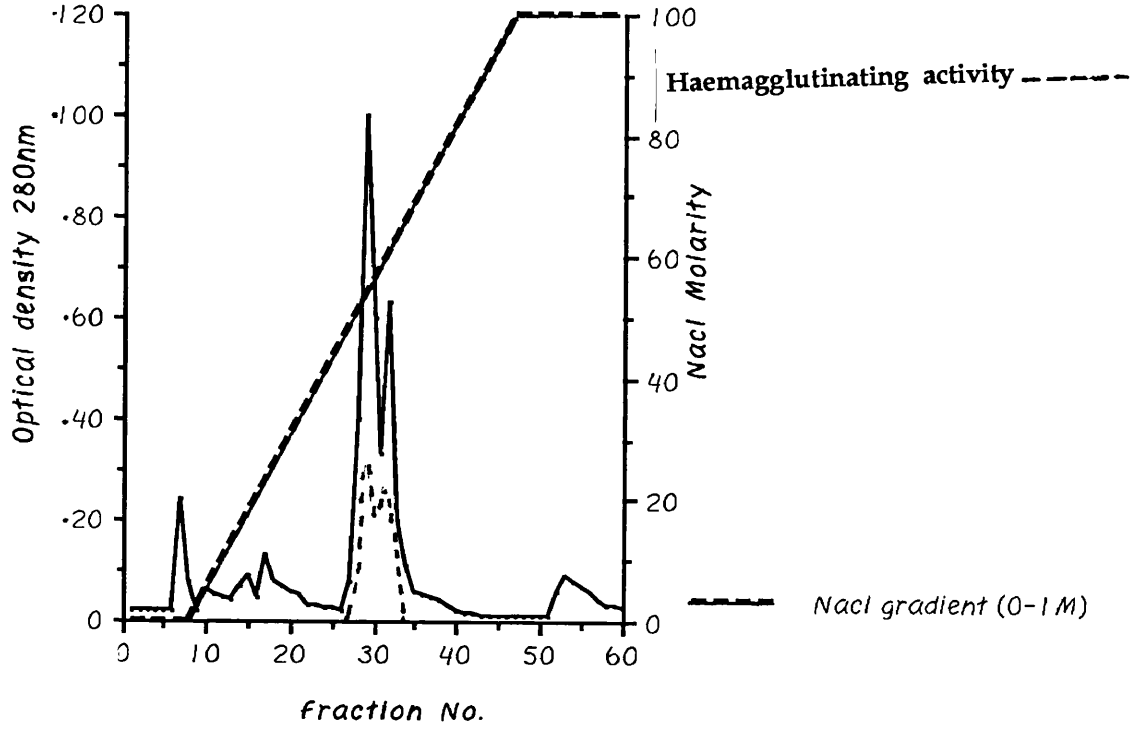
2.3.5. The effluent (the material that did not bind to the galactosyl-sepharose beads) from the galactosyl-Sepharose column was subjected to haemagglutination using RBCs, blood group B, which indicated the presence of lectin(s). This material was subjected to ion-exchange chromatography by FPLC (Wilson and Goulding, 1988).

FPLC/ion exchange chromatography is a fast protein liquid chromatography. The principle behind this technique is the same as the principle of HPLC (section 2.2.1.) but FPLC is a development from the idea of HPLC. Its main advantage is in ion-exchange chromatography. Superose 12HR gel which is agarose-based , of particle size of 10μm, that allow fast separations, high resolution and recoveries, was used. This gel (Pharmacia) was supplied prepacked for FPLC in HR 10/30 columns. The material was injected to the column, the bound material was eluted using a salt gradient (NaCl molarity 0-1M), fractions were collected and tested.

2.3.5.1. Results of FPLC/Ion-exchange

FPLC/Ion-exchange chromatography revealed the presence of 2 main peaks (Fig. 26). The fractions of both peaks were haemagglutination positive (these two peaks represent Franz Mistletoe lectin No. 2 "MLII" and Franz Mistletoe lectin No. 3 "MLIII").

Fig. 26 FPLC/Ion-exchange Chromatography of the effluent of galactosyl-Sepharose column



2.4. Identification of the 3 different lectins from Mistletoe

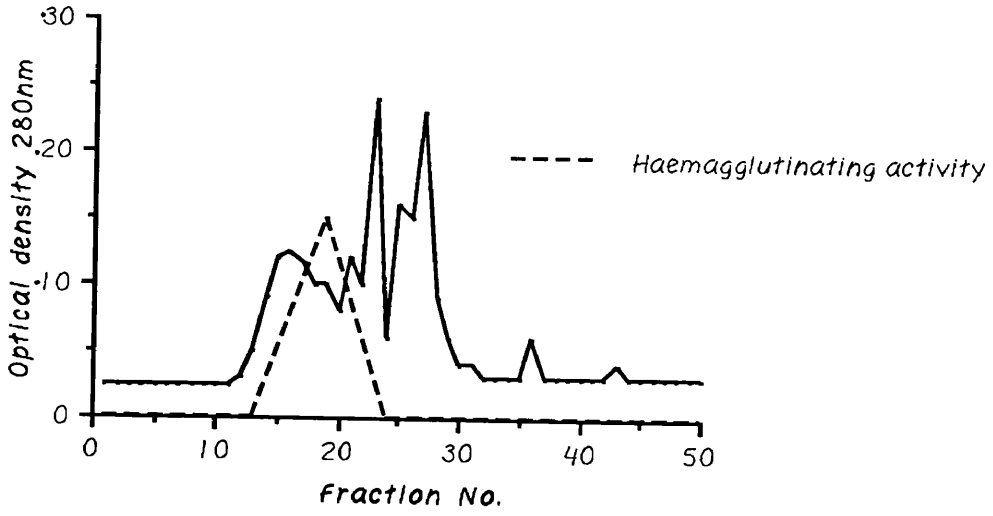
2.4.1. HPLC/Gel chromatography

MLI and the effluent from the galactosyl-Sepharose column, which was shown to contain two more different lectins by FPLC/ion-exchange chromatography, were subjected to HPLC/gel chromatography (in our lab. in London). The original crude extract was also subjected to HPLC/gel chromatography.

2.4.1.1. Results of HPLC/Gel chromatography

Gel chromatography of the crude extract revealed the presence of many peaks (Fig. 27). MLI resulted in the presence of one main peak. The fractions of this peak possessed a haemagglutinating activity (see Fig. 28).

Fig. 27



Gel chromatography of the effluent of galactosyl-sepharose material (from section 2.3.4.) gave the same results as that of the FPLC/ion-exchange and that is the presence of 2 main peaks, both were found to contain lectins (MLII and MLIII) when tested by haemagglutination (Fig. 29).

Fig. 28 HPLC/ Gel Chromatography of the affinity purified mistletoe lectin No. I

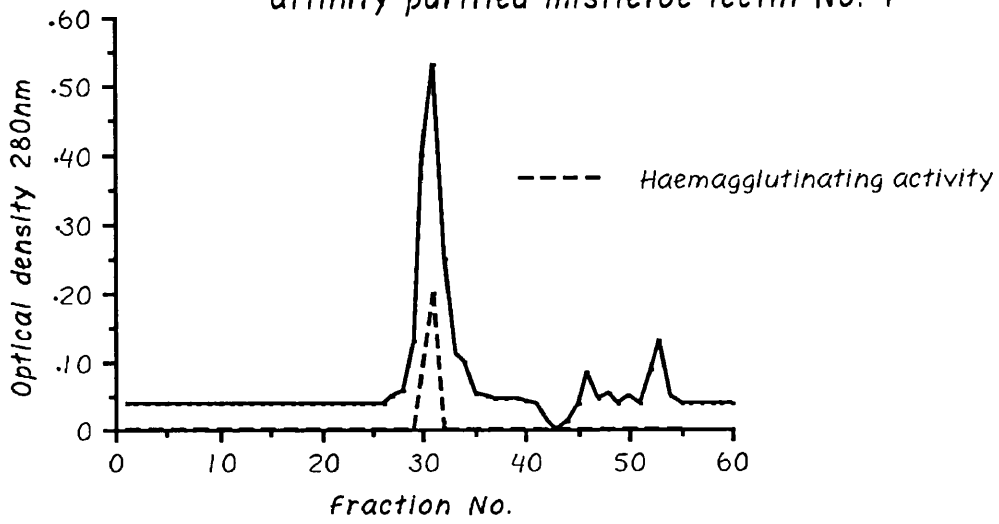
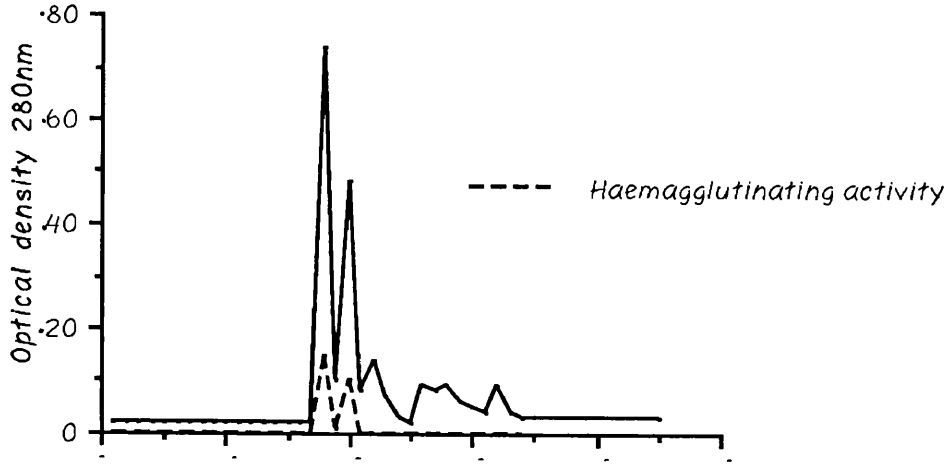


Fig. 29 HPLC/ Gel Chromatography of the effluent from the galactosyl-sepharose column



2.4.2. SDS/PAGE

2.4.2.1. SDS/PAGE (without Mercaptoethanol)

The 3 lectins were subjected to 7.5% PAGE in the presence of SDS under non-reducing conditions.

2.4.2.1.1. Results of SDS/PAGE

For MLI: A main large strong band was seen in the zone of a MW of 60KD. In addition 3 fainter bands were seen. In the zone of a MW of 34KD, 29KD and 30KD (Fig. 30).

MLII and MLIII showed a striking similarity in their MWs. They both gave a strong large band (made up of 2 chains?) of a MW of 57KD-58KD (Fig. 31).

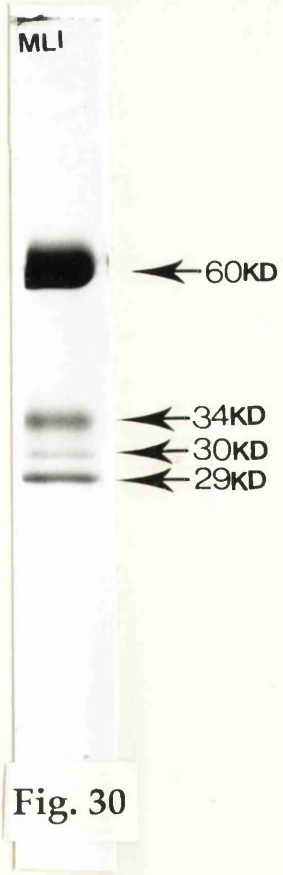


Fig. 30

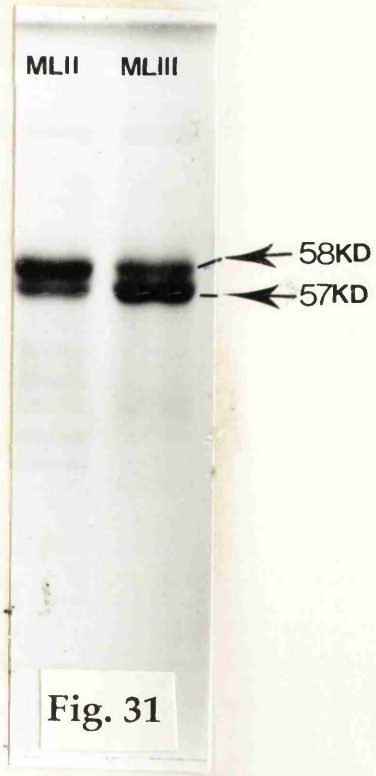


Fig. 31

Fig. 30
SDS/PAGE (7.5% gel stained with Coomassie blue, undernon reduced conditions) of MLI.
The lectin contains one major band (MW = 60KD) and 3 faint bands (MWs = 34KD, 30KD & 29KD).

Fig. 31
SDS/PAGE (7.5% gel stained with Coomassie blue, under non reduced conditions) of MLII & MLIII.
MLII contains one major band made up of 2 chains of MW of 57KD-58KD, the chain of MW of 58KD is stronger than the chain of MW of 57KD. MLIII contains one major band made up of 2 chains of MW of 57KD-58KD, the chain of MW of 57KD is stronger than the chain of MW of 58KD.

2.4.2.2. SDS/PAGE + mercaptoethanol

The 3 lectins were subjected to 12% PAGE in the presence of SDS and Mercaptoethanol.

2.4.2.2.1. Results of SDS/ PAGE (in the presence of Mercaptoethanol)

MLI gave rise to 3 bands of MW of 34KD, 29KD and 30KD (Fig. 32).

MLII gave rise to 3 main bands. They were of a MW of 27KD, 31KD and 32K, the band of MW of 31KD being fainter than the other two bands (Fig. 33).

MLIII gave the same results as MLII except that with MLIII, the band of MW of 27K being fainter than the band of MW of 32K and the band of MW of 31K (Fig. 34).

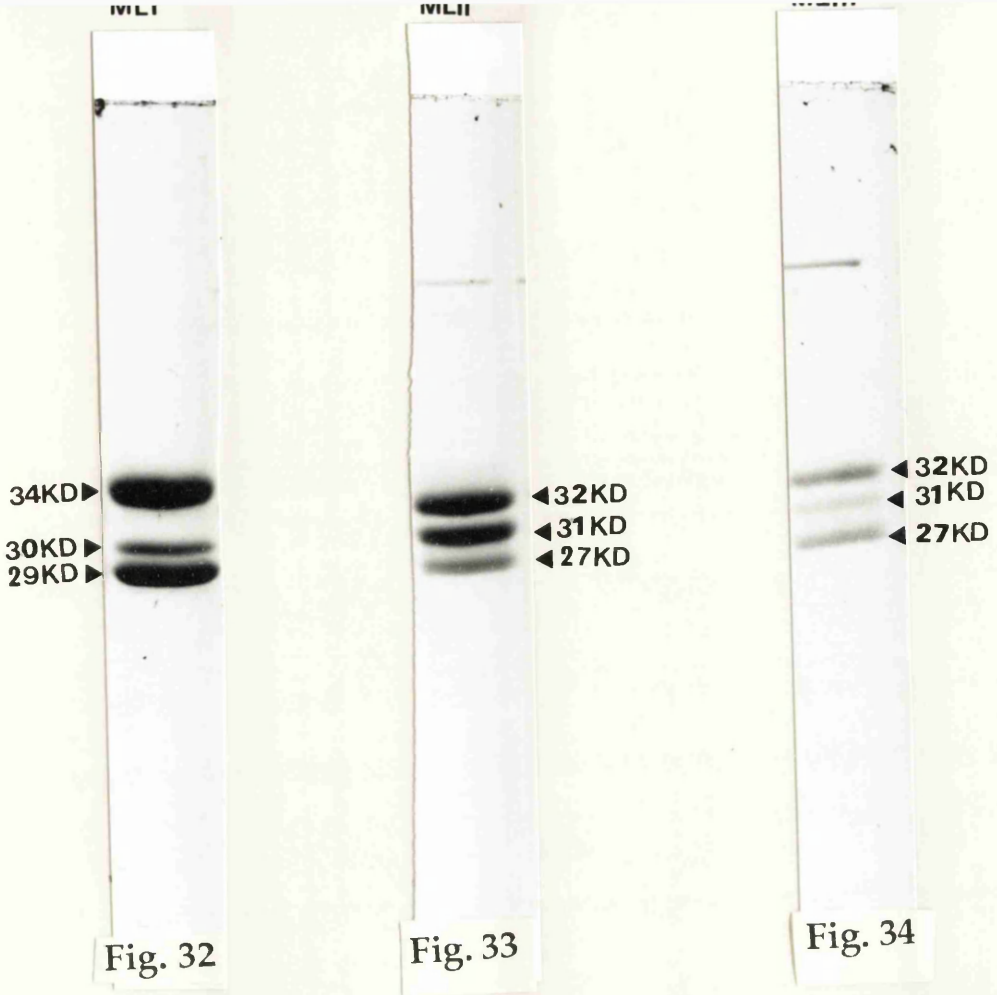


Fig. 32 Fig. 33 Fig. 34

Fig. 32
SDS/PAGE (12% gel stained with Coomassie blue, under reduced conditions) of MLI.
MLI showing 2 major bands (MW= 34KD & 29KD) and a fainter band (MW = 30KD).

Fig. 33
SDS/PAGE (12% gel stained with Coomassie blue, under reduced conditions) of MLII.
MLII showing 2 major bands (MW = 32KD & 31KD) and a fainter band (MW = 27KD).

Fig. 34
SDS/PAGE (12% gel stained with Coomassie blue, under reduced conditions) of MLIII.
MLIII showing 2 major bands (MW = 32KD & 27KD) and a fainter band (MW = 31KD).

2.4.3. Haemagglutination inhibition test

2.4.3.1. Method

Haemagglutination inhibition tests were performed for each of the 3 lectins as described before in section 2.2.3.

2.4.3.2. Results of haemagglutination test

The results are shown in Fig. 35, which indicates that MLI has strong affinity to D-Galactose and to a lesser extent to GalNAc while lectin II and lectin III have strong affinity to GalNAc and to a lesser extent to galactose.

2.4.4. A repeated haemagglutination inhibition test

This experiment was carried out to find out which of MLII and MLIII has stronger affinity to GalNAc. In this experiment 5 and 10 folds dilutions of the simple sugar were used. MLIII was found to have a stronger affinity to GalNAc than MLII . Haemagglutination with MLII was positive (not inhibited) by 0.005M GalNAc while with MLIII was positive (not inhibited) by 0.001M GalNAc.

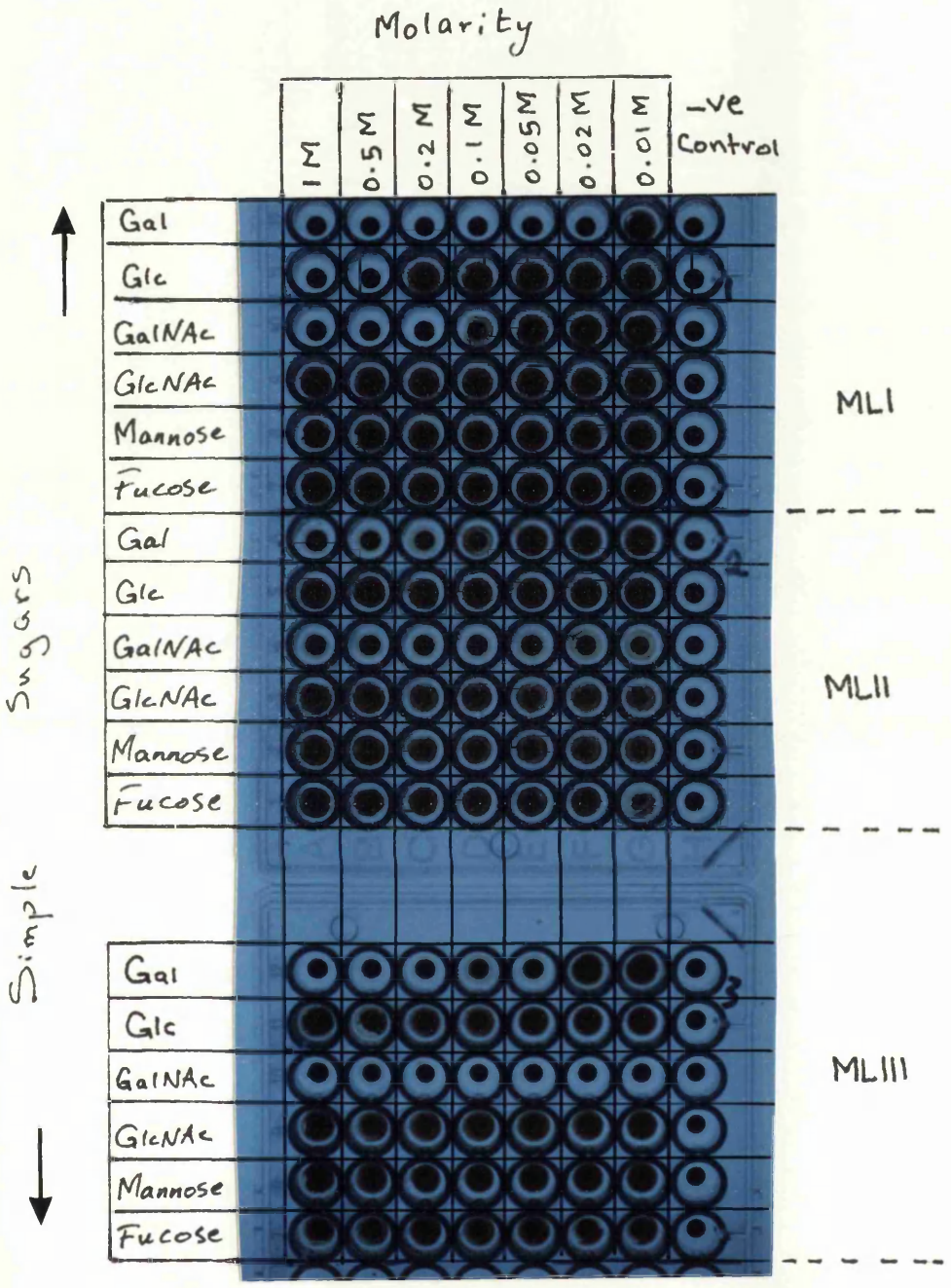


Fig. 35
Results of Haemagglutination inhibition test

2.5. Raising polyclonal antibody to MLI in rabbit

Polyclonal antibody was raised in rabbit against a purified mistletoe lectin obtained from Sigma as follows:

2.5.1. The purified lectin was treated with 1% formaldehyde in 0.1M phosphate buffer, pH 7.5, for 3 days at 37^{o}C, in order to denature the lectin, and excess formaldehyde was removed by extensive dialysis agaist 0.5M Nacl.

2.5.2. Immunization of 2 New Zealand White rabbits was initiated by subcutaneous injection of 0.5mg of protein in complete Freund's adjuvant at multiple sites, followed by 0.5mg booster doses with Freund's complete adjuvant subcutaneously every week.

2.5.3. After 6 weeks from the first immunization, the animal was bled and the serum containing the mistletoe lectin antibodies was obtained and stored at - 20^{o}C.

2.5.4. The serum was then subjected to ion-exchange chromatography by FPLC to fractionate the serum in order to obtain the Ig fraction. The anti-serum (100µl at atime) was passed on a column of Mono-Q (Pharmacia), which is DEAE, an anion exchanger. the unbound material contained the Ig fraction.

2.5.5. The Ig fraction of the serum was used in this study.

2.6. Testing the specificity of the MLI antibody

2.6.1. Ouchterlony gel double diffusion (Ouchterlony, 1962)

Principle of Ouchterlony

In this method divalent polyclonal antibody (in this case anti-mistletoe lectin antibody) crosslinks antigen (in this case mistletoe lectin), by recognizing different epitopes, and produces a precipitate.

Basically, molten agar is poured into a flat glass sheet or a Petri dish and allowed to solidify. Holes , referred to as wells, are cut in the agar. Into adjacent wells are placed antigen and antibody respectively (appendix 9). the antigen and antibody are allowed to migrate towards each other in a gel and a line of precipitation is formed where the two reactants meet. Each individual antigen produces a single precipitation line and the position of the line will be dependent upon the concentration of the antigen and its molecular weight (MW) and the concentration of antibody.

2.6.1.1. Testing the antibody specificity

The Ig fraction of mistletoe lectin antiserum (placed in the centre well) was tested against MLI, MLII, MLIII, against the crude extract and against normal rabbit sera. One well was filled with PBS as negative control.

Out of interest and also to test the lectins further, MLI, MLII & MLIII were also each tested (placed in the centre well) using this technique against normal rabbit serum, Ig fraction of normal rabbit sera and IgG fraction of the normal sera from the same animal. The lectins were also tested against human serum albumin and bovine serum albumin (BSA). As a positive control, one well was filled with Ig fraction of

mistletoe lectin antiserum (anti-ML Ig). As a negative control one well was filled with PBS only. The plates were kept overnight at 4°C before being read.

2.6.1.2. Results of Ouchterlony gel diffusion

Lines of precipitation were seen between the anti-mistletoe Ig and MLI, MLII, MLIII, and the crude mistletoe extract. No line of precipitation was seen between the anti-mistletoe Ig and normal rabbit serum or with the PBS. Interestingly, a line of precipitation was detected between MLI well and the crude mistletoe extract suggesting the presence of polysaccharides with terminal galactose in the crude extract (Fig. 36).

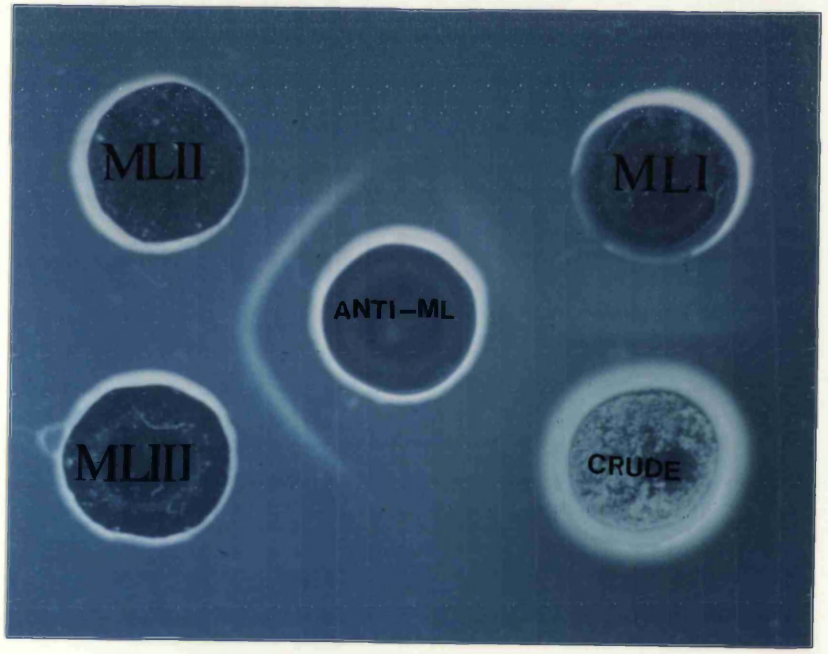


Fig. 36
Ouchterlony gel diffusion plate.
Anti-MLI, in the centre well, reacting with MLI, MLII, MLIII, & the crude extract. Note the reaction between MLI and the crude extract.

A precipitate was detected between MLI and normal rabbit serum but not with Ig fraction or the IgG fraction of the non-immune rabbit serum (Fig. 37). No precipitate was detected between MLI and human serum albumin or BSA. Similar results were obtained with MLII and MLIII

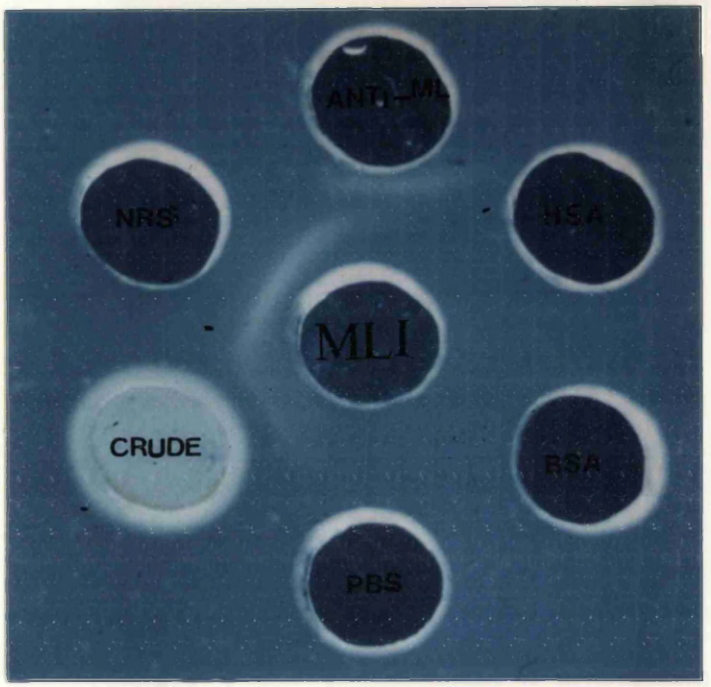
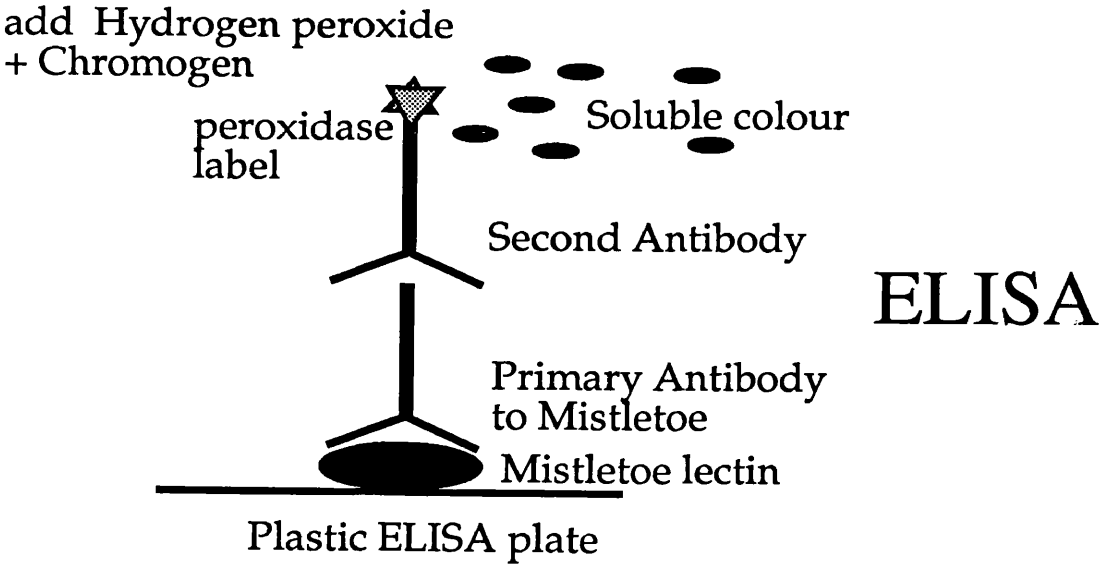


Fig. 37
Ouchterlony gel diffusion plate.
MLI reacting with anti-MLI, normal rabbit serum (NRS) and the crude extract. The line of precipitation between MLI and anti-MLI and the crude extract is continuous. Two main lines of precipitation are seen between MLI and NRS. One of these lines is continuous with the line of precipitation between MLI and anti-MLI and the crude extract.

2.6.2. Enzyme linked immunosorbent assay (ELISA) (Engvall and Pearlmann, 1971).

2.6.2.1. Principle

ELISA is an immunological technique for measuring or detecting antigen or antibody. In a test for antigen (for example), known antibody is bound to a plastic surface-usually a well in a microtitre plate, in which the subsequent reactions are carried out. An enzyme conjugated to the complementary antibody is added and the antigen/antibody reaction is visualised by incubation of the antigen/antibody/enzyme complex with a suitable substrate; this leads to development of a colour which can be measured photometrically. The most commonly used enzymes are horseradish peroxidase and alkaline phosphatase. The principle of ELISA is illustrated below in Fig. 39.



2.6.2.2. Details of the method used are presented in appendix 10

2.6.2.3 Results of ELISA

All three lectins reacted with the antibody with MLI being the strongest reactant with the antibody. The anti-mistleoe bound to MLI strongly even at dilution of 10^4 of the lectin while with MLII and MLIII the binding was weak at dilution of 10^3 of the lectins.

2.6.3. Dot-immunobinding assay (Hawkes, Niday and Gordon, 1982)

2.6.3.1. Principle of dot-immunobinding assay

The priciple of the dot immunobinding assay is as follows:

A diluted solution or suspension of antigen (in this case lectin) in saline is "dotted" on to a nitrocellulose membrane (NCM). and the dot then incubated with the test antibody. After blocking the nonspecific antibody binding sites on the paper, antibody binding is detected either by peroxidase conjugated (in this case) or a peroxidase-conjugated second antibody directed against the first antibody used. After the development of the peroxidase, a positive reaction is detected as a coloured dot against the white membrane background (Fig. 40).

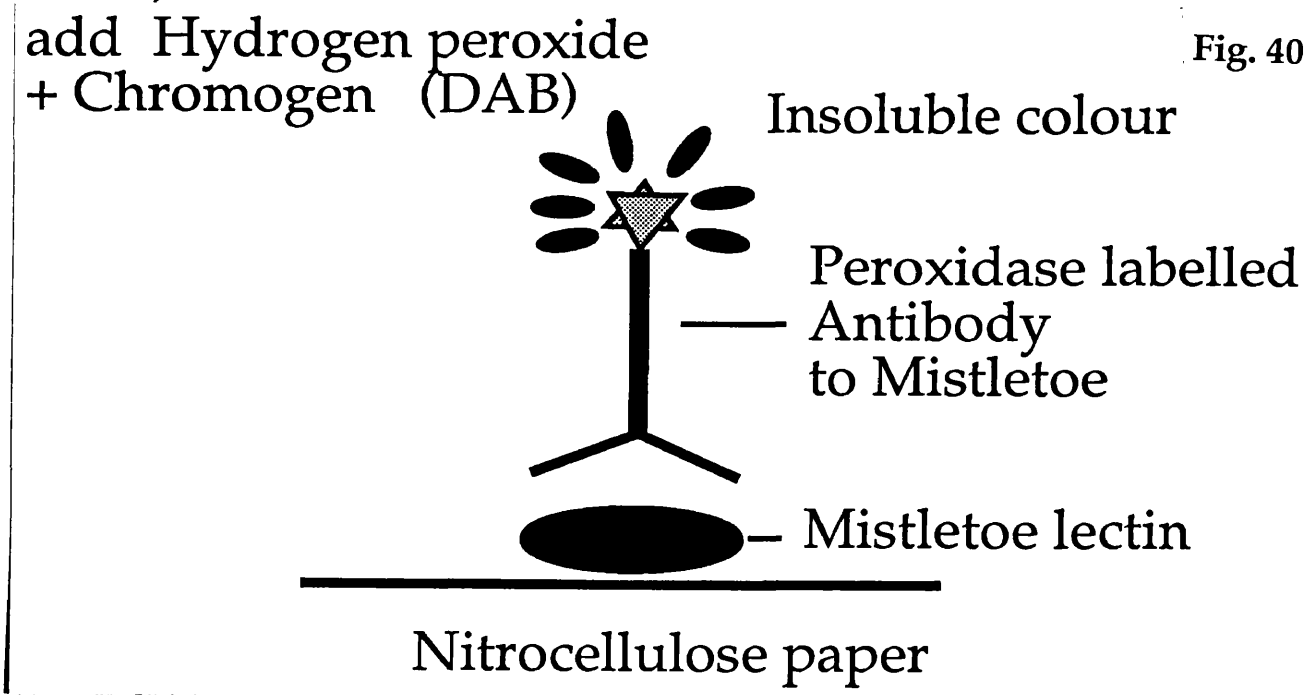


Fig. 40

2.6.3.2. A typical protocol is given in appendix 11

2.6.3.3. Results of dot-immunobinding assay

The anti-MLI Ig reacted with all the 3 mistletoe lectins. However, the reaction was stronger with MLI. No staining was obtained in the negative control. The optimal dilution of the antibody in this system was found to be 1:200 (Fig. 41)

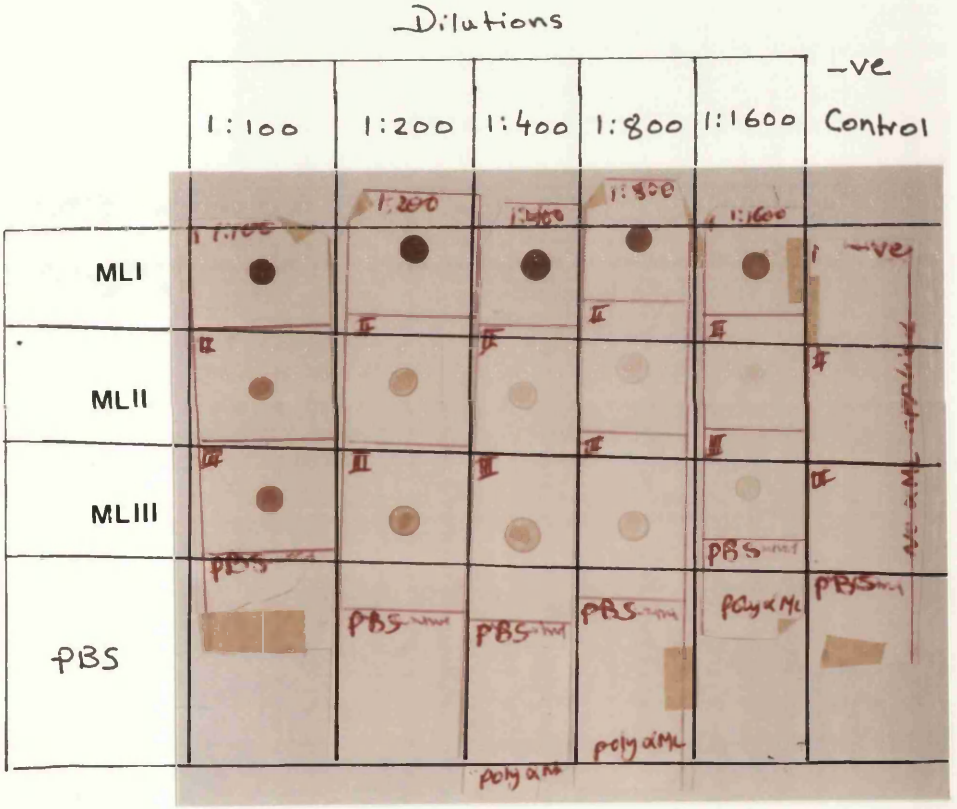


Fig. 41
Dot-immunobindinding assay on nitrocellulose membrane.
The anti-MLI reacted with the 3 lectins. The reaction is the strogest with MLI.

2.6.4. Western blotting (Kyhse-Andersen, 1984)

2.6.4.1. Principle of western blotting

Protein blotting is the electrophoretic transfer of proteins from polyacrylamide gels to a nitrocellulose membrane in a way that a faithful replica of the original pattern is obtained. The method results in quantitative transfer of the original bands with no loss of resolution. In the semi-dry method the gel to be transfered is sandwiched between wet layers of filter papers which are in turn sandwiched between two solid graphite plates as electrodes. Transferring proteins onto NCM allows detection of proteins by immunological procedures. All additional binding capacity on the NCM needs to be blocked with excess protein; then a specific antibody bound and finally the binding sites developed by peroxidase reaction product.

2.6.4.2. Western blotting of mistletoe lectins

MLI, MLII & MLIII were first subjected to PAGE in the presence of SDS under non-reducing conditions (Mercaptoethanol was not included in the sample buffer). The 3 lectins were also subjected to PAGE in the presence of SDS under reducing condition (Mercaptoethanol was included in the sample buffer). One gel of each experiment was stained with Coomassie blue stain and a duplicate gel of each was used for protein blotting. Proteins from each gel then were transferred to a NCM using semi-dry multi gel electroblotter (ancos) (appendix 13).

2.6.4.3. Results of SDS-PAGE and the Western blotting

The anti-mistletoe immunoglobulin showed binding to the 3 lectins in

this method and also to their chains (Fig. 42, 43, 44 & 45). Additional bands were detected by the immunoperoxidase staining on NCM that were not seen in the gel stained by Coomassie blue stain. Presumably this is because of the high sensitivity of the immunoperoxidase staining compared with the sensitivity of the Coomassie blue level of detection.

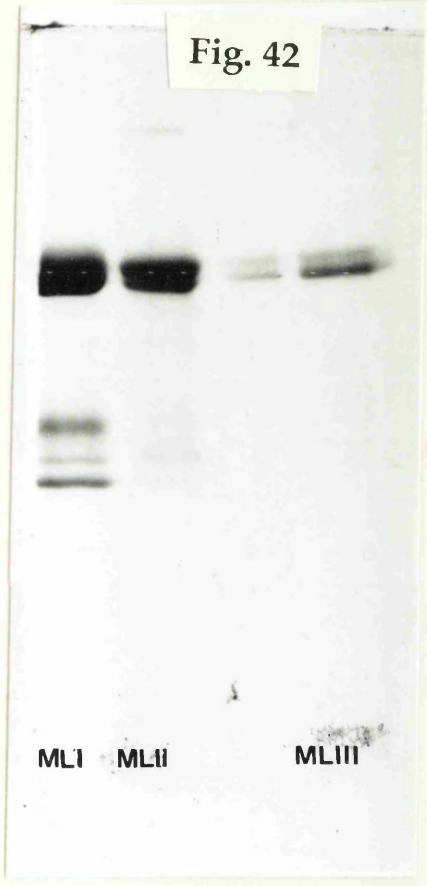


Fig. 42
SDS/PAGE (7.5% gel run under non-reduced conditions and stained with Coomassie blue) for MLI, MLII & MLIII.

Fig. 43
An identical gel to the one seen in Fig. 42 blotted on NCM and stained by direct immunoperoxidase method.
The anti-MLI reacted with the 3 lectins.

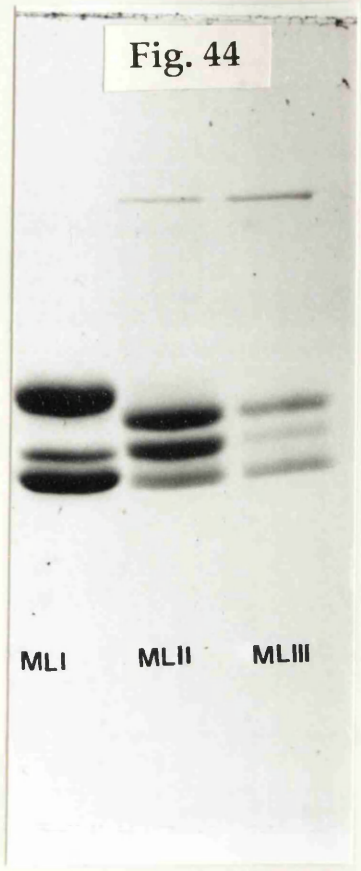


Fig. 44
SDS/PAGE (12% gel run under reduced conditions and stained with Coomassie blue) for MLI, MLII & MLIII.

Fig. 45
An identical gel to the one seen in Fig. 44 blotted on NCM and stained by direct immunoperoxidase method for lectins binding. The anti-MLI reacted with the chains of the lectins. Note the presence of additional bands (arrowed) not seen in the gel stained with Coomassie blue. These extra bands correspod to MW of 60KD in MLI and to MW of 58KD in MLII.

The main aim of this study was to explore the underlying relationship between Mistletoe lectins and cancer cells, by seeking binding of these lectins to formalin-fixed paraffin-embedded primary breast cancer tissues, and then to correlate the binding of cancer cells to clinical behaviour of these cancers.

The following section describes the application of Mistletoe lectins to cancer tissues and the results .

3. TISSUE BINDING TO LECTINS

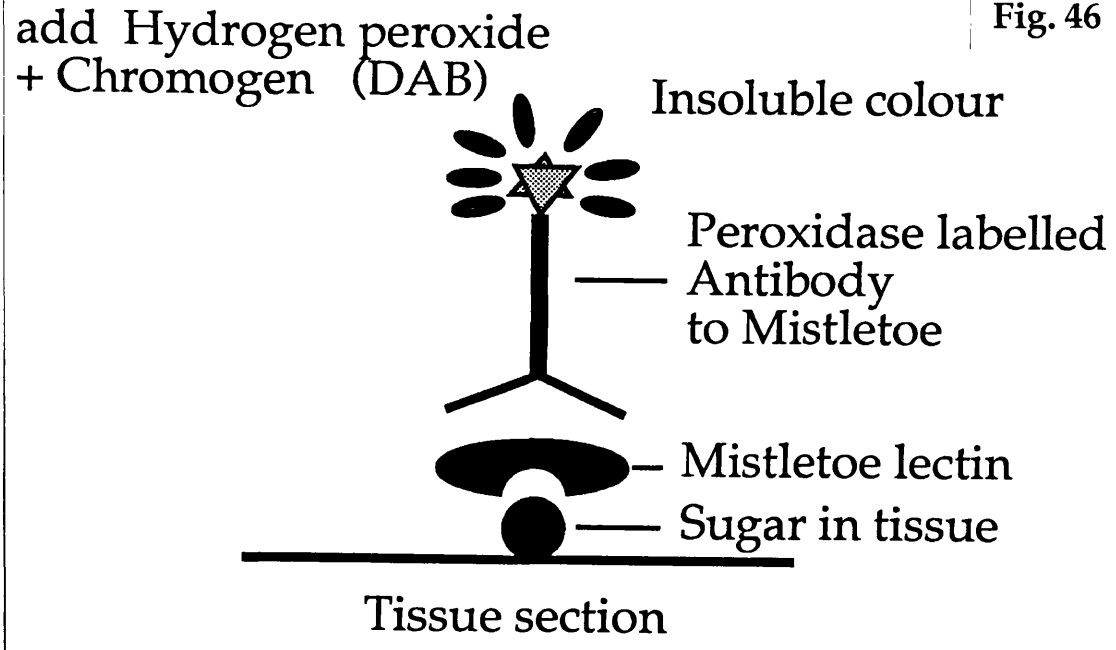
3.1. Tissues

A total of 234 cases of primary breast cancer were stained for MLI, MLII and with MLIII. These represent all 193 primary breast cancer cases, where tissues were available, presented at the Bland-Sutton Institute, Middlesex Hospital between 1978 and 1980 plus 41 miscellaneous primary breast cancer cases presented between 1981 and 1985.

3.2. Lectin-peroxidase staining

The principle of the staining method (shown below in Fig. 46) is outlined below. To detect lectins binding to tissue receptors the lectin is directed to a tissue section where it incubated and will bind to what is presumed to be carbohydrate(s). These are refered to as lectin-receptors. The binding of lectin to receptor could be visualized by directing an antibody to the lectin(s) that has been itself labeled to an enzyme (peroxidase in this case).

Fig. 46



3.2.1. Determination of optimal conditions

Initially the formalin-fixed paraffin-embedded tissues of primary breast cancer cases were stained with MLI, MLII & MLIII, using the following lectin-peroxidase method to determine the optimal dilution of the (1) lectins and (2) the antibody conjugate.

3.2.1.1. Lectin-peroxidase staining method No. 1 (details of the method is in appendix 14):

Paraffin sections of primary breast cancer were dewaxed. Endogenous peroxidases were blocked with H_2O_2 / methanol. The tissues were then incubated with each of the 3 Mistletoe lectins at different dilutions (6ug/ml-200µg/ml in TBS). This was followed by rabbit anti-ML Ig labelled with peroxidase (POD/Anti-ML) at different dilutions (3µg/ml-100µg/ml in TBS). The binding sites were visualized using H_2O_2/DAB (appendix 12).

Negative controls

Two types of negative controls were used:

1- Omitting the application of the lectin, instead the sections were left in TBS. To detect non- specific binding to POD/Anti-ML.

2- Omitting the application of POD/Anti-ML to detect residual endogenous enzyme (peroxidase)

Result of staining method No. 1

For MLI, the optimal dilution (highest specific staining with least "non-specific staining") was found to be 100µg/ml followed by POD/Anti-ML at dilution of 10µg/ml.

For MLII, the optimal dilution was found to be 50 µg/ml followed by

POD/Anti-ML at dilution of 10μg/ml.

For MLIII, the optimal dilution was found to be the same as for MLII, 50 μg/ml followed by POD/Anti-ML at dilution of 10μg/ml.

Since sections of primary breast cancer cases that were stained with the lectins often showed the presence of background staining, in some cases the interpretation of the results was difficult. The following was tried.

3.2.1.2. Use of Albumin to reduce non-specific background staining

Since MLs bind to normal swine serum and normal rabbit serum (as shown by Ouchterlony immunodiffusion method in section 2.6.1.2.), bovine serum albumin (BSA) was used to dilute the lectin. The negative controls where the lectins were omitted, were treated with BSA instead. Albumin was used to block non-specific binding since it does not appear to express sugars that bind to Mistletoe lectins (MLs) (as shown by Ouchterlony immunodiffusion method in section 2.6.1.2.). Because the background staining made interpretation difficult, adjacent sections of the same cancer cases were stained (staining method No. 2) using the same staining method as in section 3.2.1.1. , bu the only difference between the two methods was the inclusion of BSA in diluting lectins So, the lectins were diluted in 3% (BSA) in TBS.

Negative controls

Omitting the application of the lectin, instead the sections were treated with 3% BSA in TBS.

Result of staining method No. 2

In the sections that were stained for the 3 lectins, the inclusion of the BSA not only reduce the non- specific staining (background staining) (Fig 47, 48 & 49) but inhanced the specific staining (Fig. 50 , 51 & 52). In cases where the interpretation of the results was difficult when the previously mentioned method was used, after the inclusion of BSA, the results were clearer to read (Fig. 53, 54 & 55).

The negative controls where the lectin was omitted but instead treated with 3% BSA in TBS then with POD/Anti-ML, were completely negative.

Fig. 47 Primary breast carcinoma stained with H & E. X 40

Fig. 48 Same case as in case 47 stained for MLI. BSA was not included. Note the presence of non-specific background staining. X40

Fig. 49 Same case as in Fig. 47 stained for MLI. BSA was included. Note the reduction of the background staining. X40

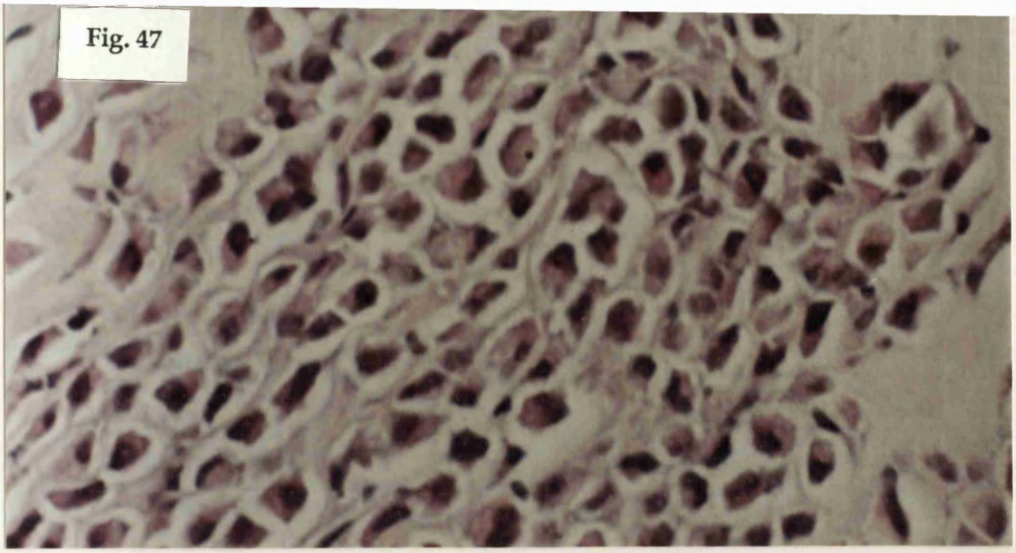


Fig. 47

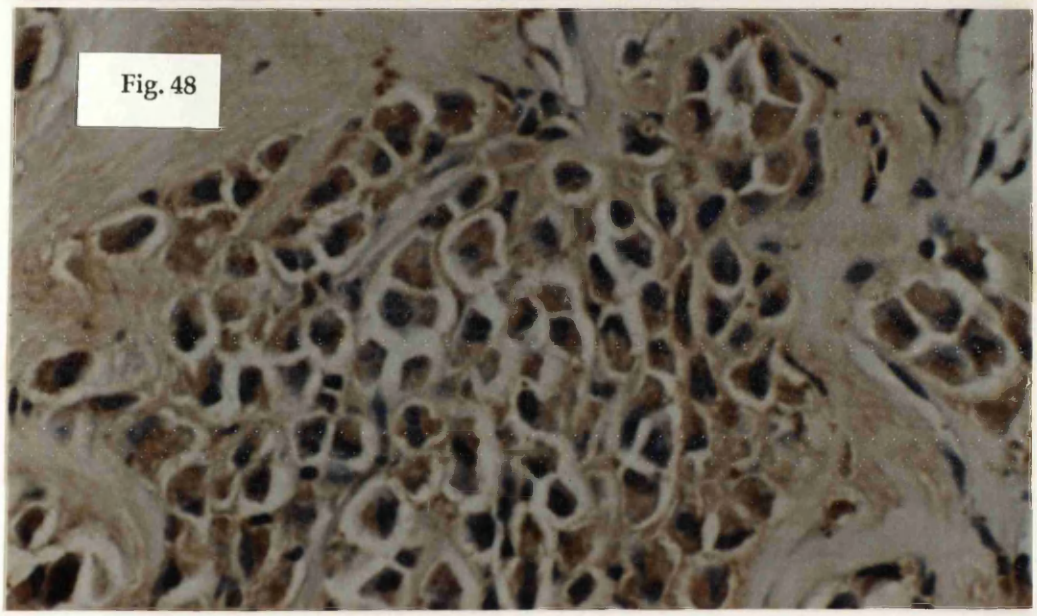


Fig. 48

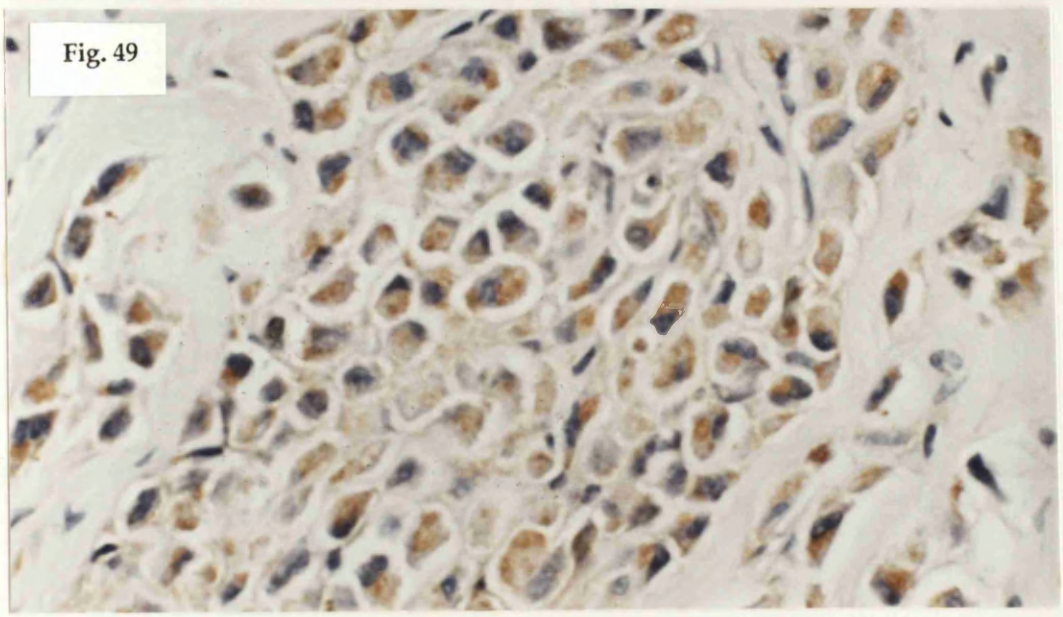
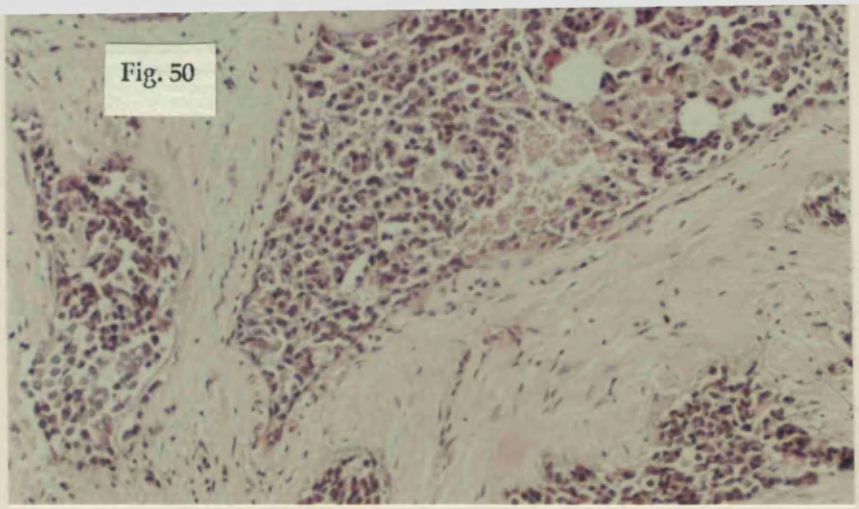
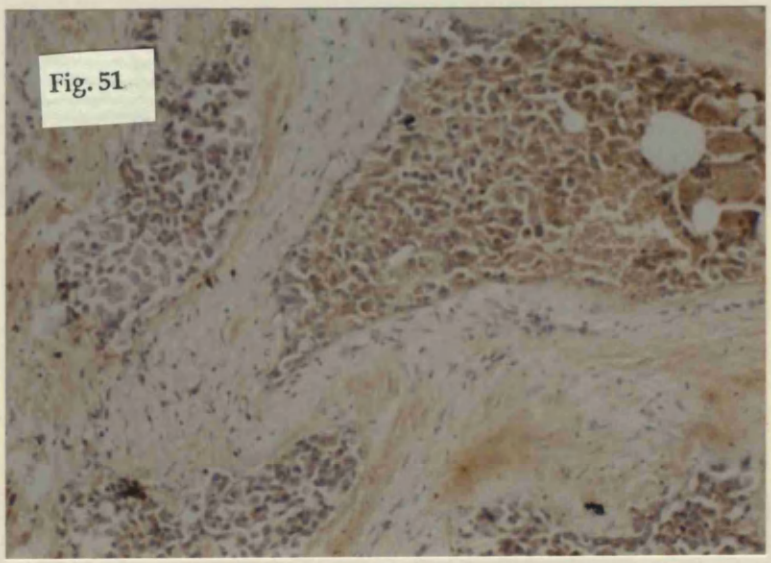


Fig. 49





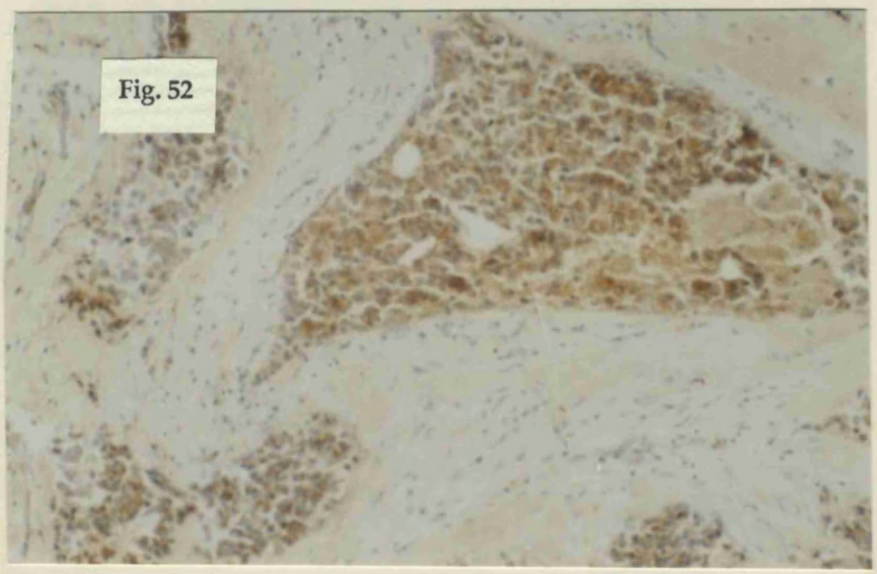


Fig. 50 Primary breast carcinoma stained with H & E. X 20

Fig. 51 Same case as in Fig. 50 stained for MLII. BSA was not included. X 20

Fig. 52 Same case as in Fig. 50 stained for MLII. BSA was included and has enhanced the specific staining. X 20

Fig. 53 Primary breast carcinoma stained with H & E. X 40

Fig. 54 Same case as in fig. 53 stained for MLIII. BSA was not included. It is to difficult to assess with certainity whether the case is positive or negative for MLIII. X 40

Fig. 55 Same case as in Fig. 53 stained for MLIII. BSA was included. Tumour cells are clearly negative. X 40

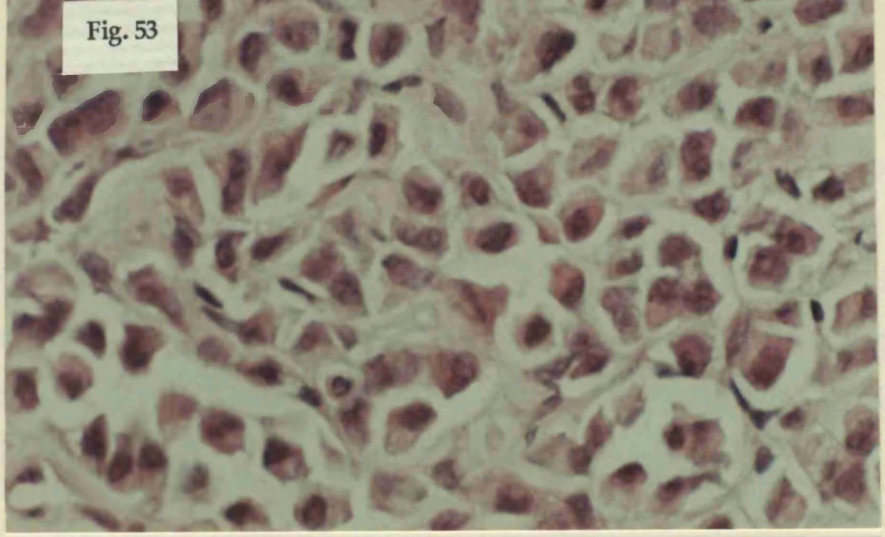


Fig. 53

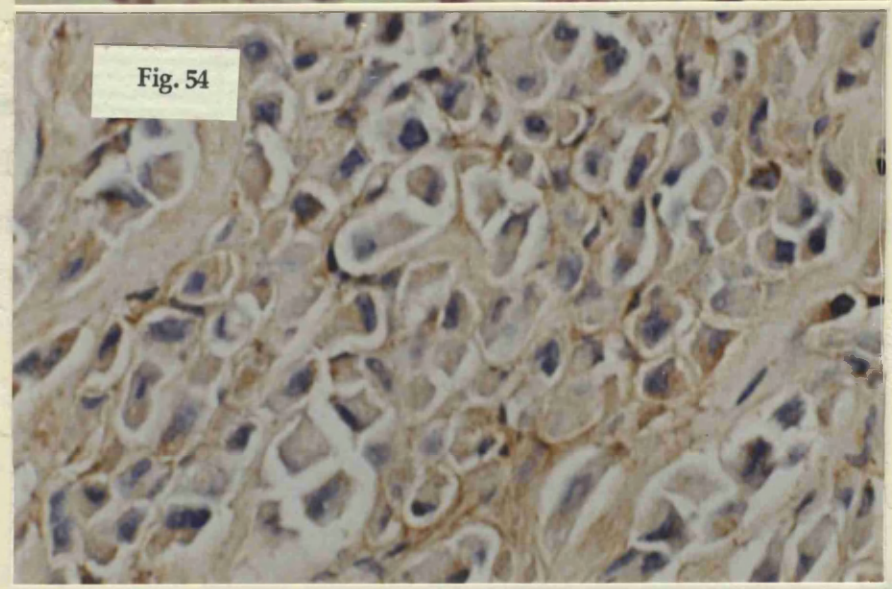


Fig. 54

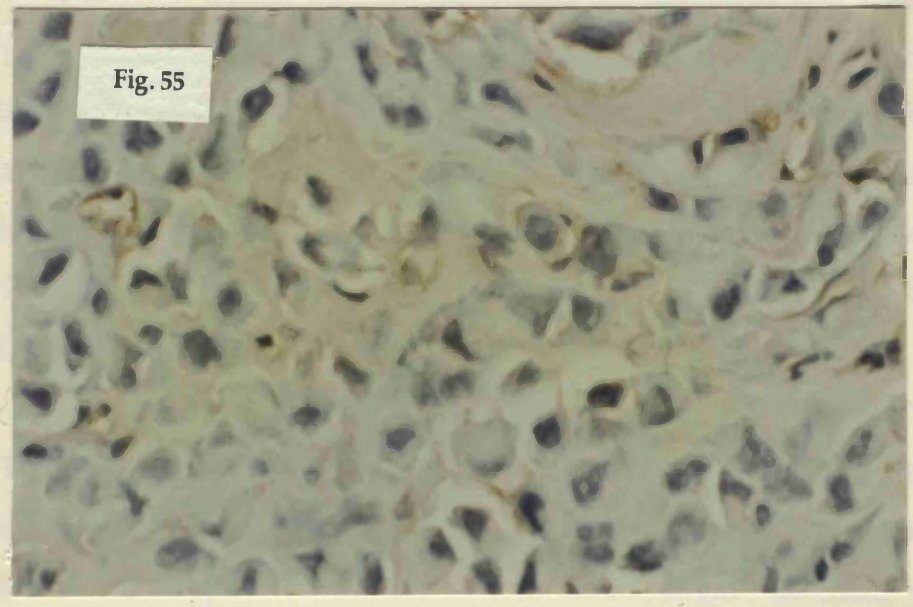


Fig. 55

3.2.1.3. Enzyme treatment

Trypsin effect on staining was also determined. The sections were treated with Trypsin solution (400mg trypsin + 400mg Calcium chloride were dissolved in 400ml TBS prewarmed to 37°C in a glass dish) at 37°C for 5 minutes, 10 minutes, 15 minutes and for 20 minutes. Trypsin treatment of the sections was carried out after blocking the endogenous peroxidase, before incubation with the lectins.

Results of trypsinization

No difference was seen with trypsinization in all the different times tried. No enhancement of the positive staining. Sections that were negative before trypsin treatment stayed negative after this treatment.

3.2.1.4. Tissue-binding inhibition test by simple sugars

Since the Mistletoe lectins showed an affinity for galactose and GalNAc and in order to find out if what the lectins bind to on sections are the carbohydrate that the lectins have affinities to, known positive sections of primary breast cancer were stained using the optimized staining method, for the 3 lectins. Adjacent sections of the same cancer cases were treated, instead of the lectin solution, with lectins that have been incubated with 0.5M sugar that the lectin has an affinity to.

Each lectin was incubated with galactose, GalNAc, a mixture of galactose and GalNAc, and Mannose as control. The sections were stained with the lectins in their optimal dilution. The lectin-sugar mixture was prepared in a way that the lectin would be diluted with the 0.5M sugar to make the lectins in their optimal dilution.

Results of binding inhibition test

Incubation with mannose had no inhibitory effect on the binding of any of the 3 Mistletoe lectins. Galactose had a strong inhibitory effect on the binding of MLI and to a lesser degree on the binding of MLII and MLIII. GalNAc had some inhibitory effect on the binding of MLI but it had a very strong inhibitory effect on binding of MLII and MLIII. A mixture of galactose and GalNAc had a strong inhibitory effect on the binding of the 3 lectins. None of these carbohydrates completely abolished the reactivity of the tissues with lectins or completely inhibited the binding of these lectins to their tissue receptors.

3.2.2. Interpretation of staining results

The staining of primary breast cancer cases was scored as positive (+ve), negative (-ve) or unknown. The scoring of cases as -ve or +ve was based on the staining of cancer cells. No quantitation of positive staining was done.

3.2.2.1. Staining of cancer cells

1. Positive cancers

The cases were considered +ve when cancer cells showed definite brown staining. The carcinomas could be divided into three categories on the basis of positive staining reaction with mistletoe lectins (MLs):

(a) those in which all or the majority of tumour cells showed evidence of binding to MLs;

(b) carcinomas in which there were mixtures of positive and negative cells, either intermingled or in clusters; and

(c) tumours in which only a small number of cells (10%-20%) reacted.

A variable intensity of staining between cancer cells was also found in some cases. Details of pattern of staining are described on page 132.

Continued overleaf

2. Negative cancers

When no brown staining at all was detected in malignant cells and the cells were completely -ve, or when trace staining was detected, or when a very faint diffuse and negligable amount of staining was present, the cases were considered -ve.

3. Unknown staining results

When a number of cells showed staining which was not as definite as in +ve cases yet at the same time not easy to call negative, the staining was repeated. When the repeated staining revealed the same pattern, the cases were then cautiously entered as unknown. These 17 cases are described on page 131.

3.2.2.2. Staining of non-malignant components

Positivity of endothelium of blood vessels and of erythrocytes was seen in many tissue sections (see Figs 83-85).

The staining of lymphocytes and monocytes was also noted (see Figs 83-85).

MLs also stained many normal and benign epithelial components noted in tissue sections examined and gave a uniform staining: diffuse cytoplasmic and/or luminal surface staining of epithelial cells lining both lobules and ducts (staining was seen in both ductal and myoepithelial cells) (see Figs 93-95).

The staining of blood vessles, lymphocytes, monocytes, normal and benign breast cells was unrelated to staining of tumour cells (see Figs 98-100).

3.3. Results of Staining with MLI, MLII & MLIII

A total of 234 cases were stained for MLI, MLII and for MLIII.

MLI

Out of the 234 cases stained for MLI:

130 (55.5%) cases were +ve

87 (37.1%) cases were -ve

17 (7.2%) cases were unknown.

MLII

Out of the 234 cases stained for MLII:

135 (57.6%) cases were +ve

83 (35.4%) cases were -ve

16 (6.8%) were unknown

MLIII

Out of the 234 cases stained for MLIII:

132 (56.4%) cases were +ve

86 (36.7%) cases were -ve

16 (6.8%) cases were unknown.

MLI, MLII & MLIII

Out of the 234 cases stained for MLI, MLII & MLIII:

120 (51%) cases were +ve for the 3 lectins at the same time.

3.4. Patterns of breast cancer tissue staining with MLI, MLII & MLIII

Where the same cases were positive for the 3 lectins, the localization of the binding was almost always the same with one difference is the degree of reactivity; binding was either cytoplasmic (Fig. 56, 57, 58, 59 & 60) and/or on the cell borders (Fig. 61, 62, 63, 64 & 65).

Some cases were negative for MLI while positive for MLII and for MLIII (Fig. 66, 67, 68, 69 & 70). Few cases were +ve for MLI while negative for MLII & MLIII. All the cases (except 3 cases) that were positive for MLII were positive for MLIII.

Nearly half the cancer cases tested for Mistletoe lectins binding showed no reactivity to any of the Mistletoe lectins (Fig. 71, 72, 73, 74 & 75).

In some cases lymphocytes stained in addition to cancer cells (Fig. 76, 77, 78, 79 & 80).
In some cases lymphocytes were -ve while cancer cells were +ve (Fig. 81, 82, 83, 84 & 85).
Where in some cases lymphocytes stained positive while cancer cells negative (Fig. 86, 87, 88, 89 & 90), these cases were considered negative.

Benign duct epithelial cells showed faint cytoplasmic positivity with strong reactivity on the luminal surface, for the 3 lectins while in the same cases malignant cells showed heterogeneity in binding ,i.e., not all malignant cells in positive sections were positive; there were negative cells and also the degree of reactivity was not the same (Fig. 91, 92, 93, 94 & 95).
Where in some cases benign ducts stained positive while cancer cells were negative (Fig. 96, 97, 98, 99 & 100), these cases were considered negative.

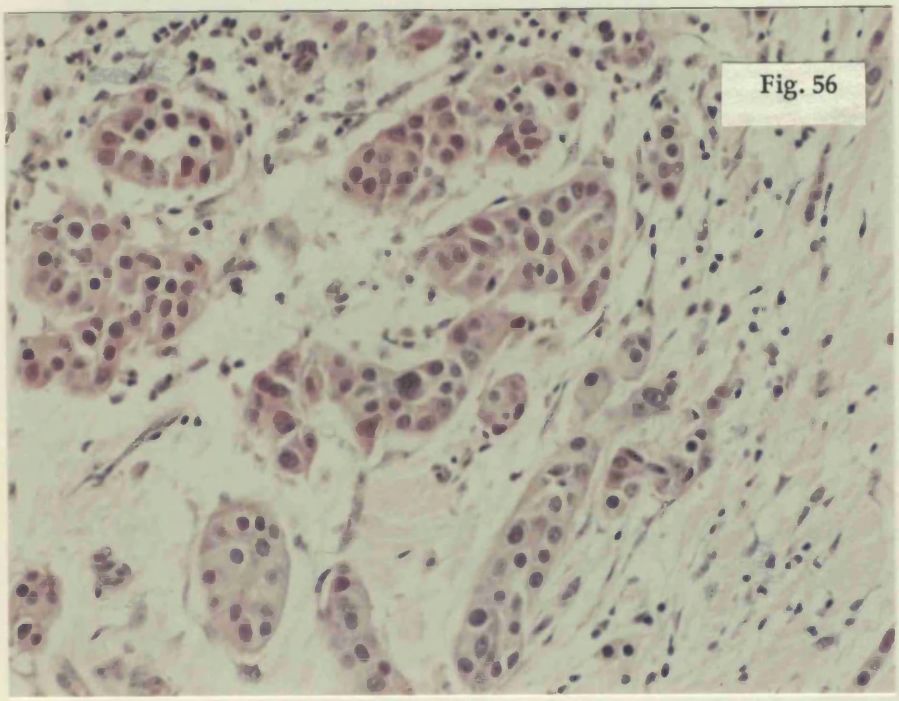


Fig. 56 Section of primary breast carcinoma stained with H & E. The section was of mixed ductal and lobular infiltrating carcinoma. X 20

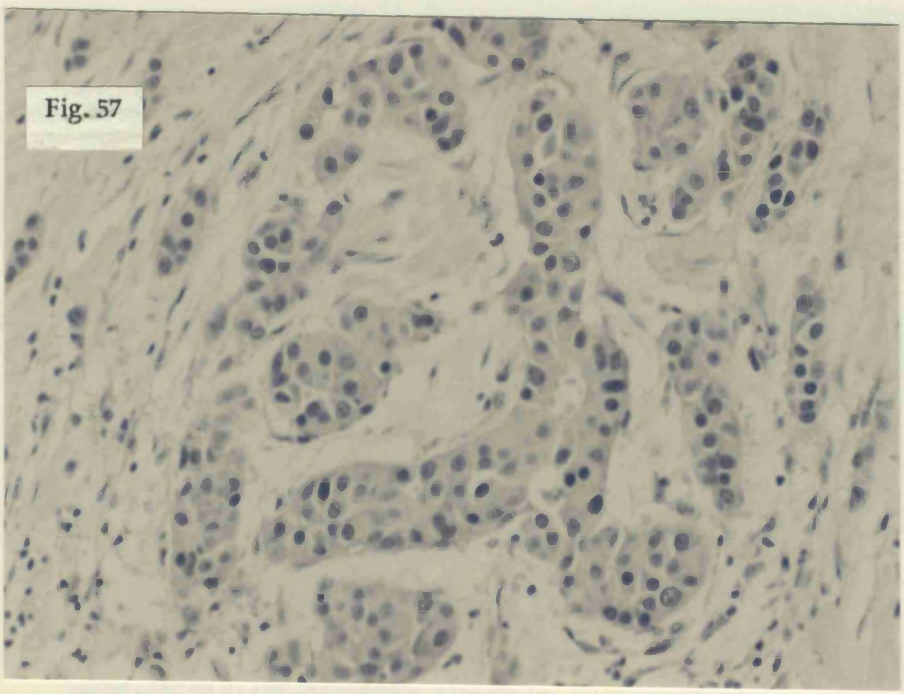


Fig. 57 Same case as in Fig. 56. Negative control (No lectin was applied to the section, the section was treated with BSA instead). X 20

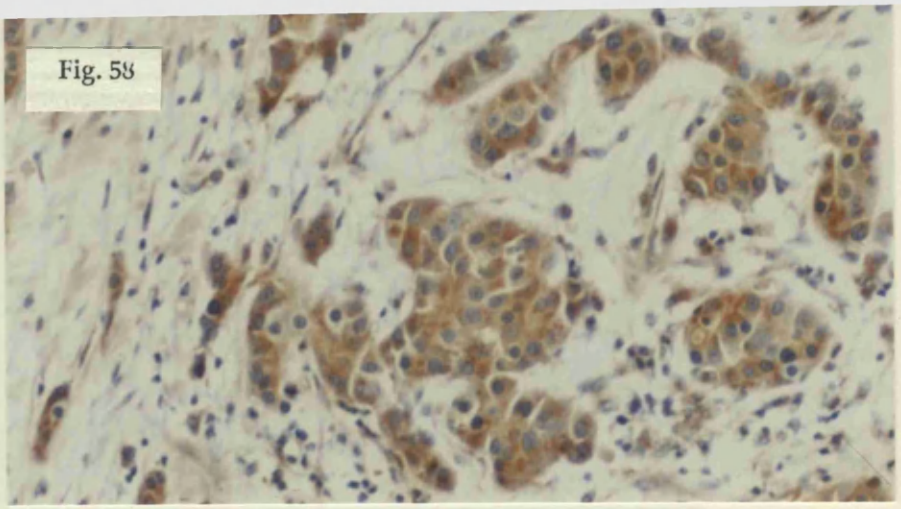


Fig. 58

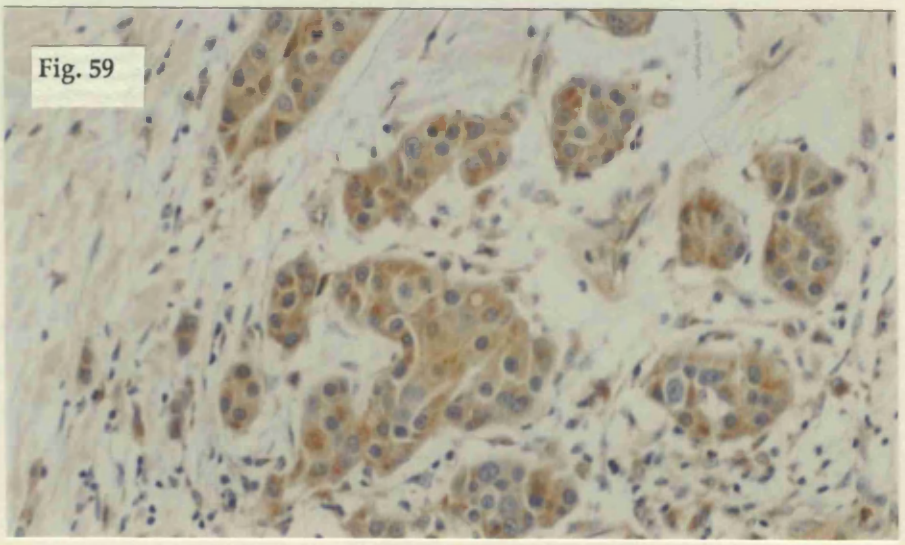


Fig. 59

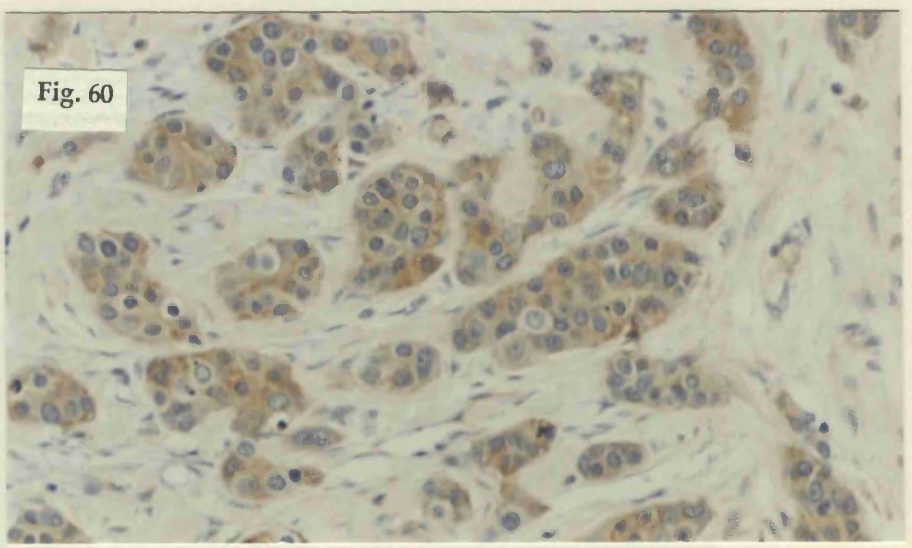


Fig. 60

Fig. 58 Same case as in Fig. 56, stained with MLI.

Fig. 59 Same case as in Fig. 56, stained with MLII.

Fig. 60 Same case as in Fig. 56, stained with MLIII.

The malignant cells are showing cytoplasmic positivity for the 3 lectins. The reactivity with MLI is stronger than that with MLII and MLIII. The reactivity with MLII is stronger than that with MLIII.

X 20

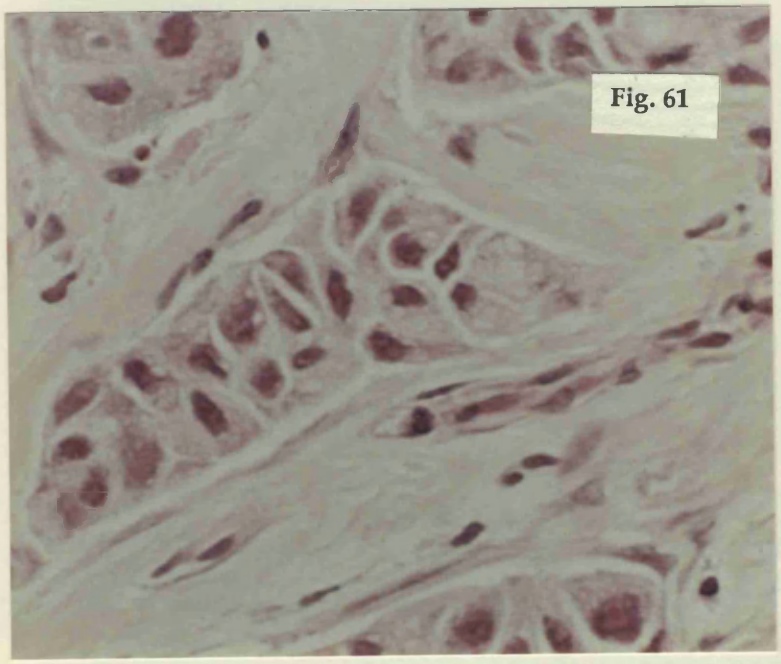


Fig. 61 Section of primary breast carcinoma stained with H & E. The section was of poorly differentiated ductal carcinoma.
X40

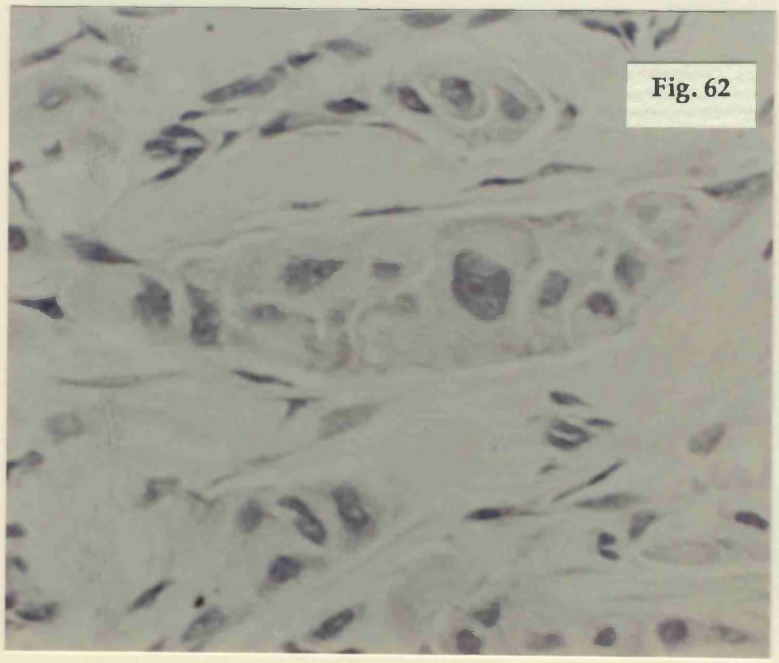
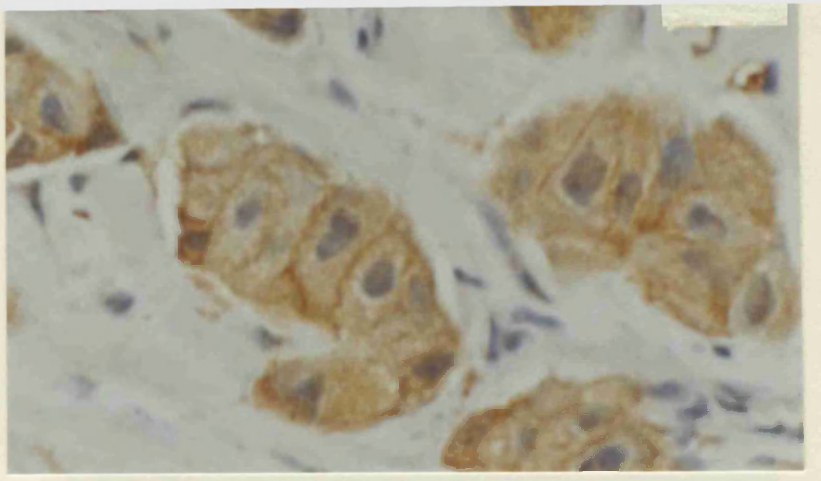
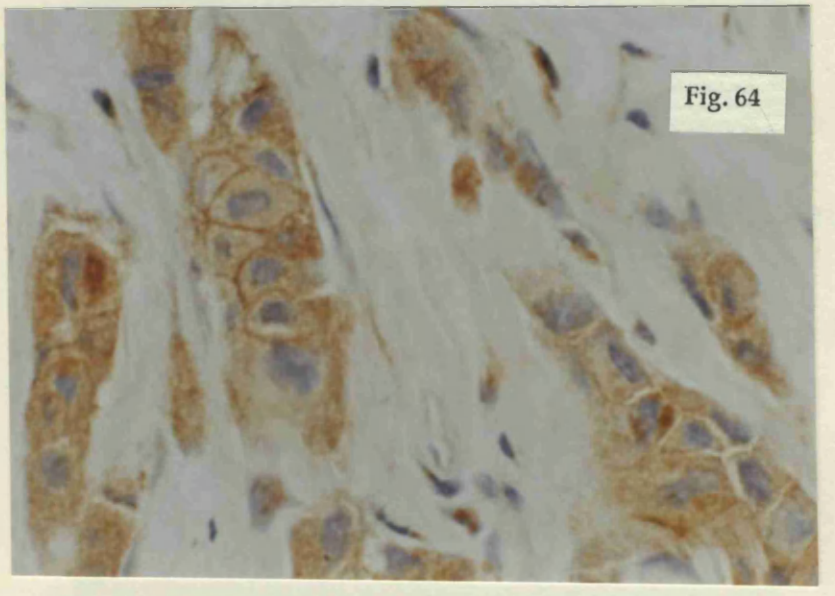


Fig. 62 Same case as in Fig. 61. Negative control. No staining was seen when omitting the application of lectins.
X40





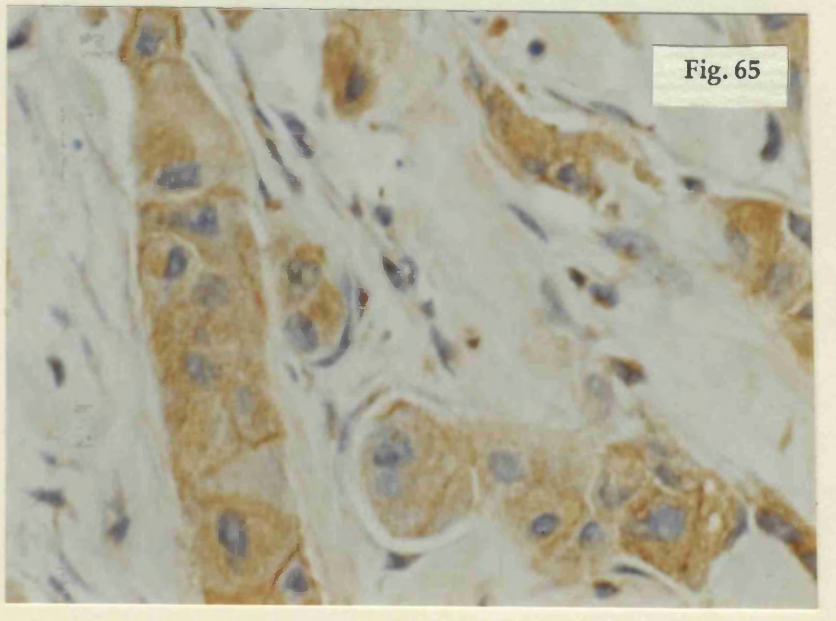


Fig. 63 Same case as in Fig. 61, stained with MLI.

Fig. 64 Same case as in Fig. 61, stained with MLII.

Fig. 65 Same case as in Fig. 61, stained with MLIII.

Cancer cells are showing weak cytoplasmic positivity and/or condensation of the positivity on the cell borders for the 3 lectins.

X 40

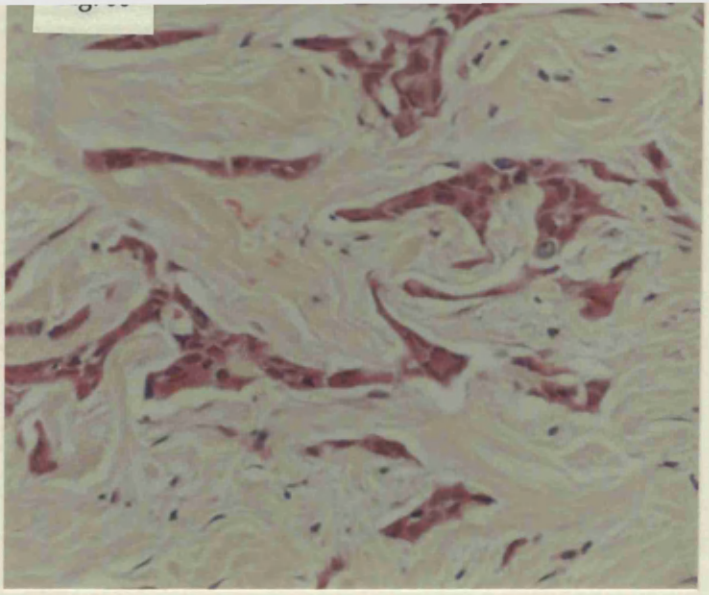


Fig. 66 Section of primary breast carcinoma stained with H & E. The section was of infiltrating ductal carcinoma.
X 20

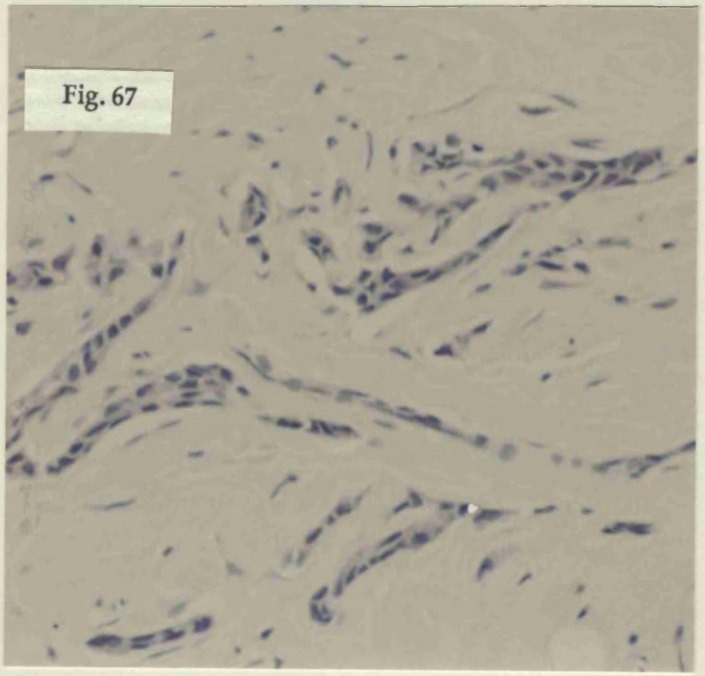
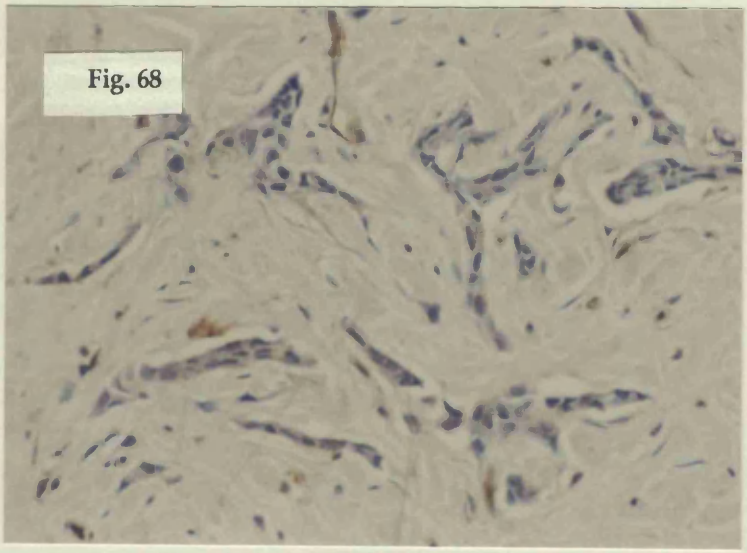
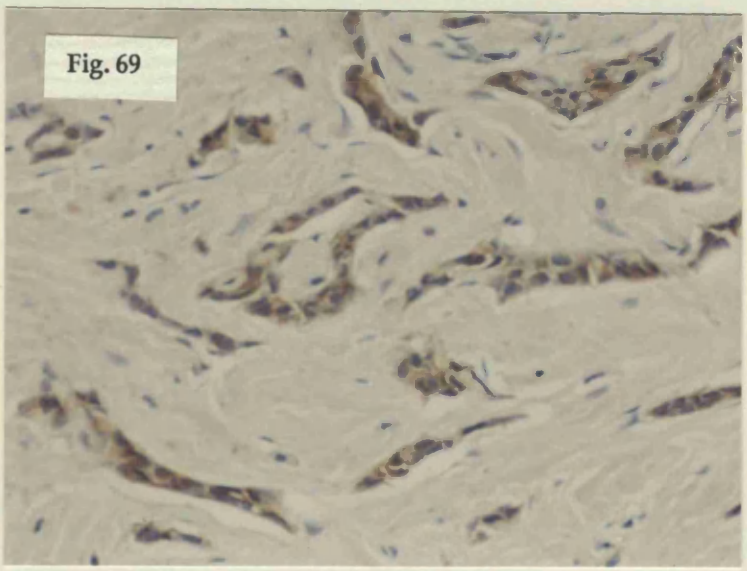


Fig. 67 Same case as in Fig. 66. Negative control.
No staining is present.
X 20





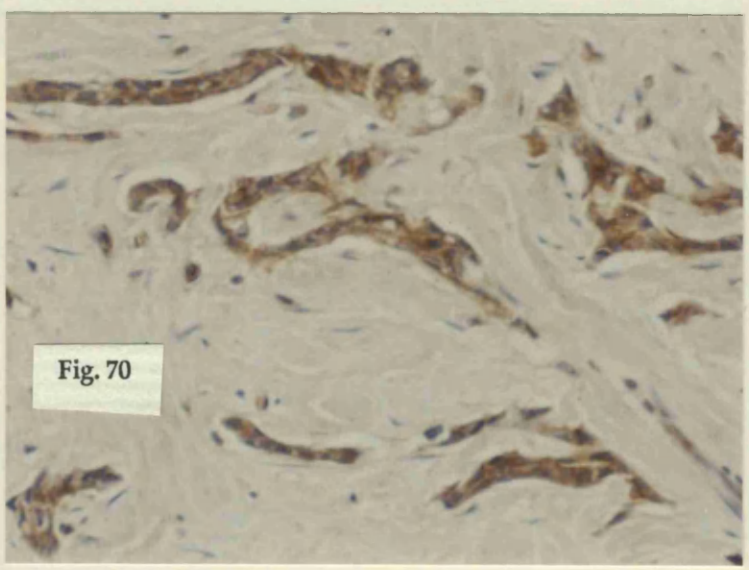


Fig. 68 Same case as in Fig. 66, stained with MLI.

Fig. 69 Same case as in Fig. 66, stained with MLII.

Fig. 70 Same case as in Fig. 66, stained with MLIII.

The malignant cells are showing lack of reactivity with MLI. The same malignant cells reacted with MLII and stronger with MLIII.

X 20

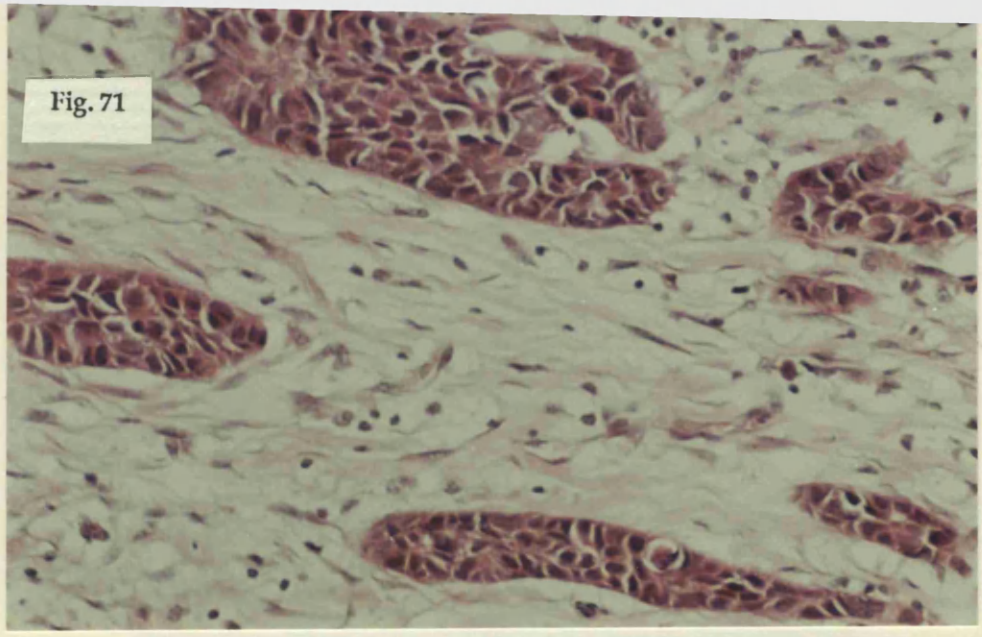


Fig. 71 Section of primary breast carcinoma stained with H & E. The section was of poorly differentiated ductal carcinoma.
X 40

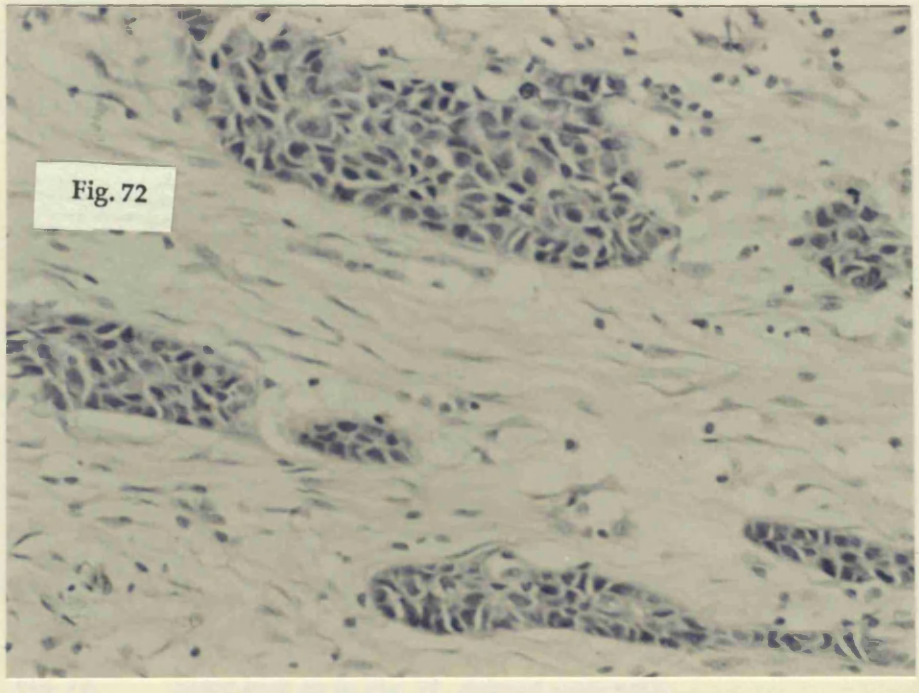
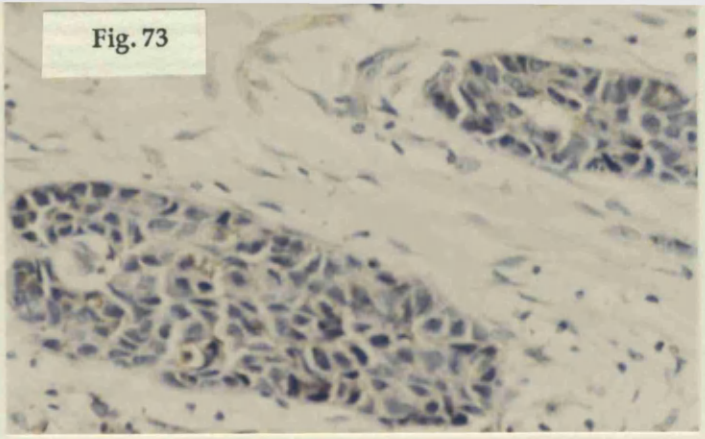
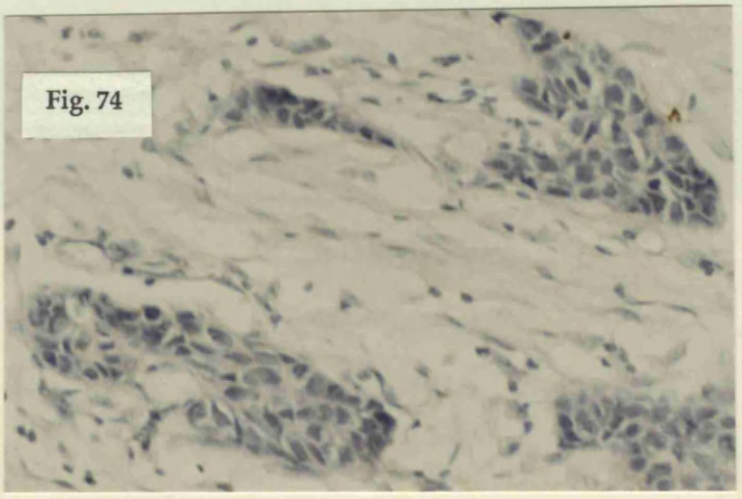


Fig. 72 Same case as in Fig. 71. Negative control.
Omitting the lectins resulting in the section being completely negative.
X 40





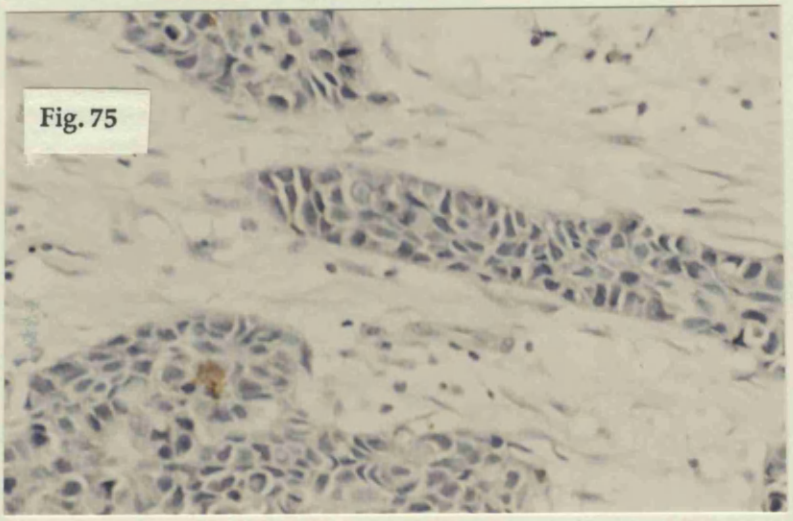


Fig. 73 Same case as in Fig. 71, stained with MLI.

Fig. 74 Same case as in Fig. 71, stained with MLII

Fig. 75 Same case as in Fig. 71, stained with MLIII

The malignant cells are -ve for the 3 lectins.

X 40

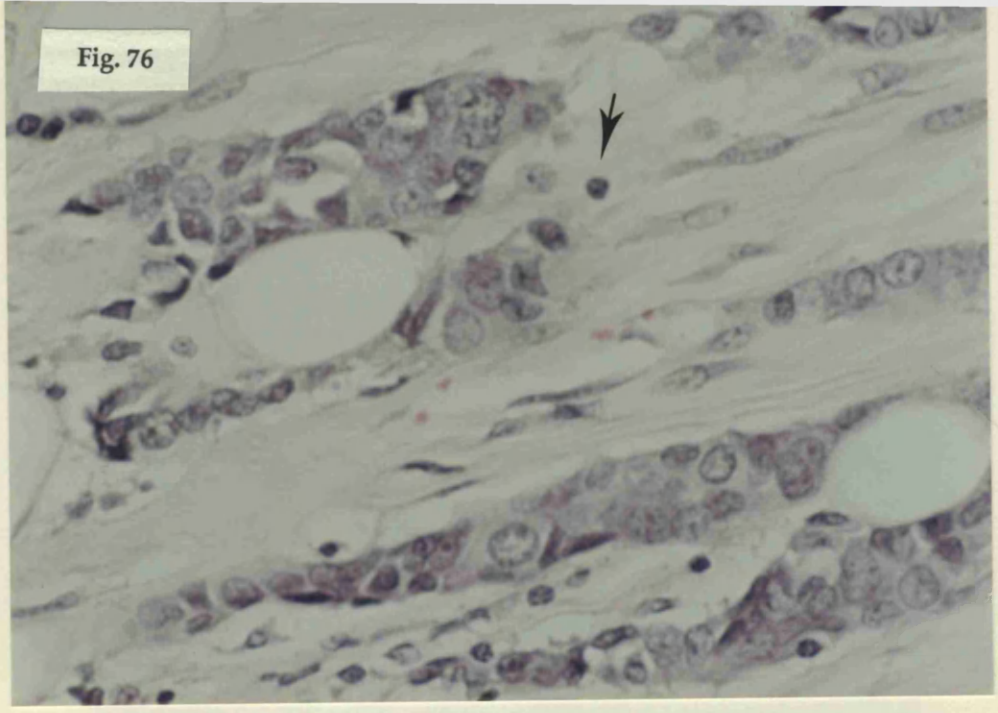


Fig. 76 Section of primary breast carcinoma stained with H & E.
Note the presence of scattered lymphocytes (arrowed). The case was of polygonal cell carcinoma.
X 40

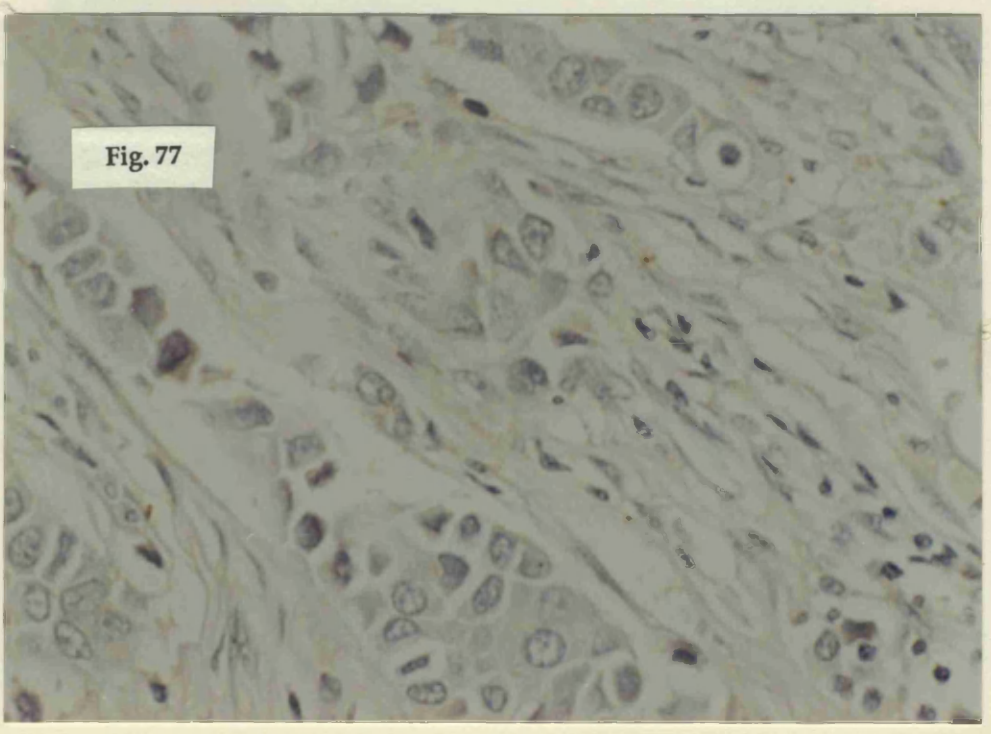
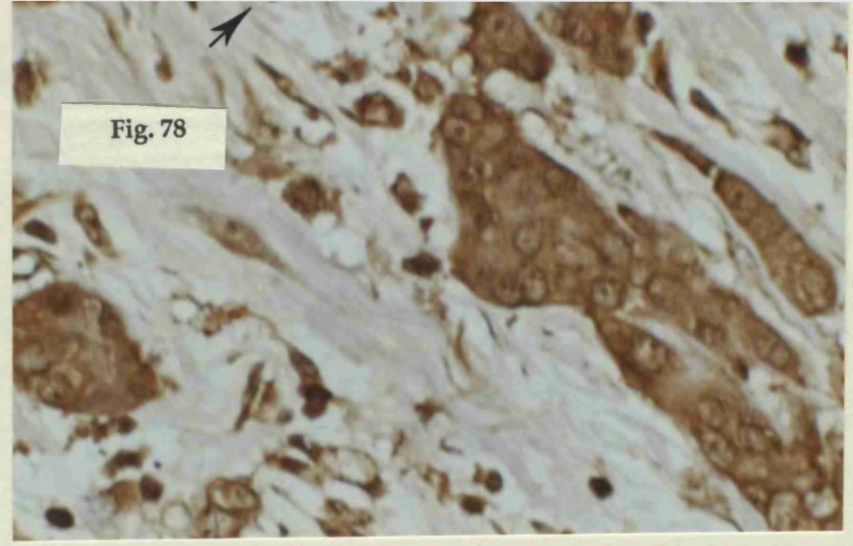
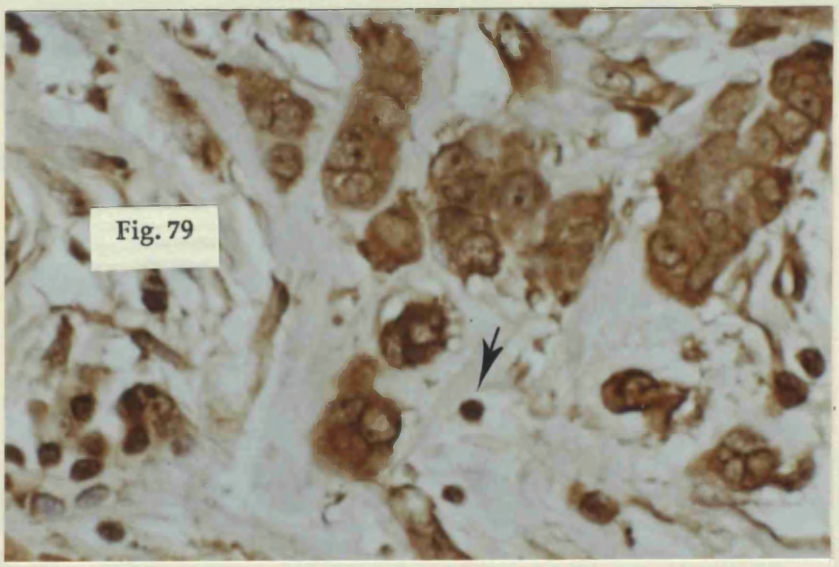


Fig. 77 Same case as in Fig. 76. Negative control.
No staining of cancer cells or lymphocytes.
X 40





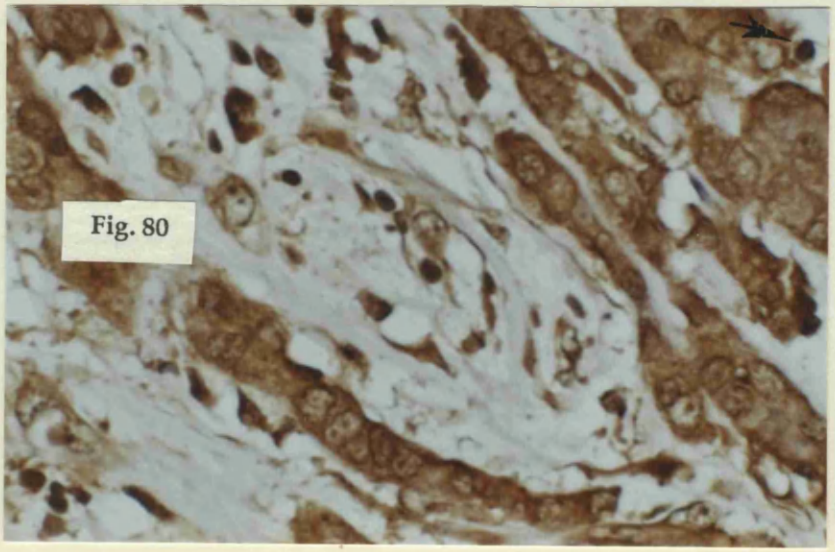


Fig. 78 Same case as in Fig. 76, stained with MLI.

Fig. 79 Same case as in Fig. 76, stained with MLII.

Fig. 80 Same case as in Fig. 76, stained with MLIII.

Both cancer cells and lymphocytes (arrowed) stained strongly for MLI, MLII, & MLIII. The staining is mainly cytoplasmic. Condensation on cell borders is also seen.

X 40

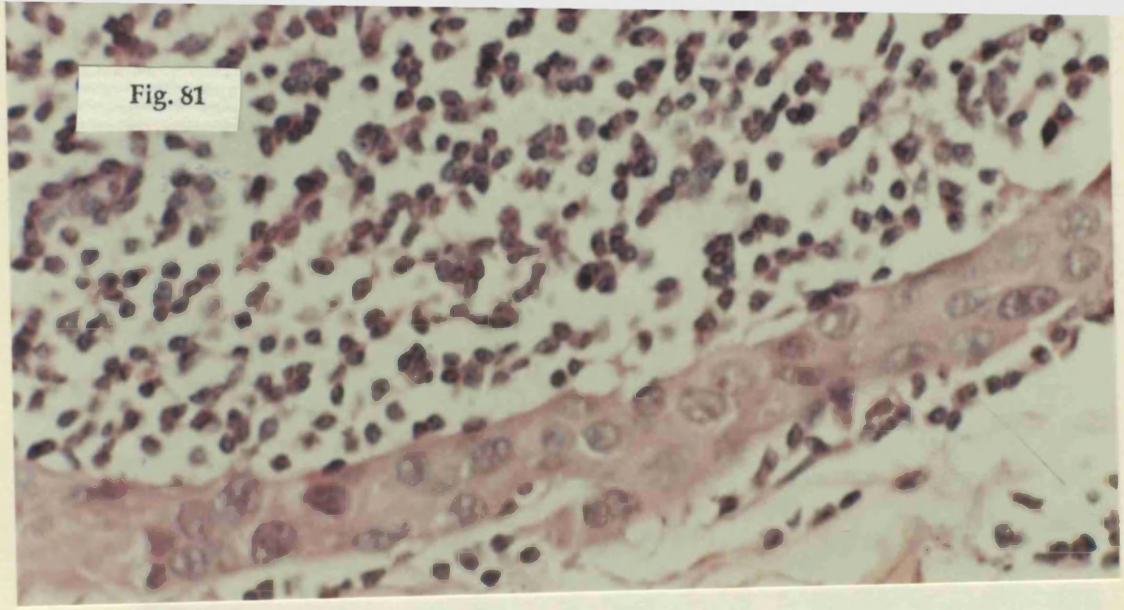


Fig. 81 Section of primary breast carcinoma stained with H & E. The section was of ductal infiltrating carcinoma. Large number of lymphocytes is present.
X 40

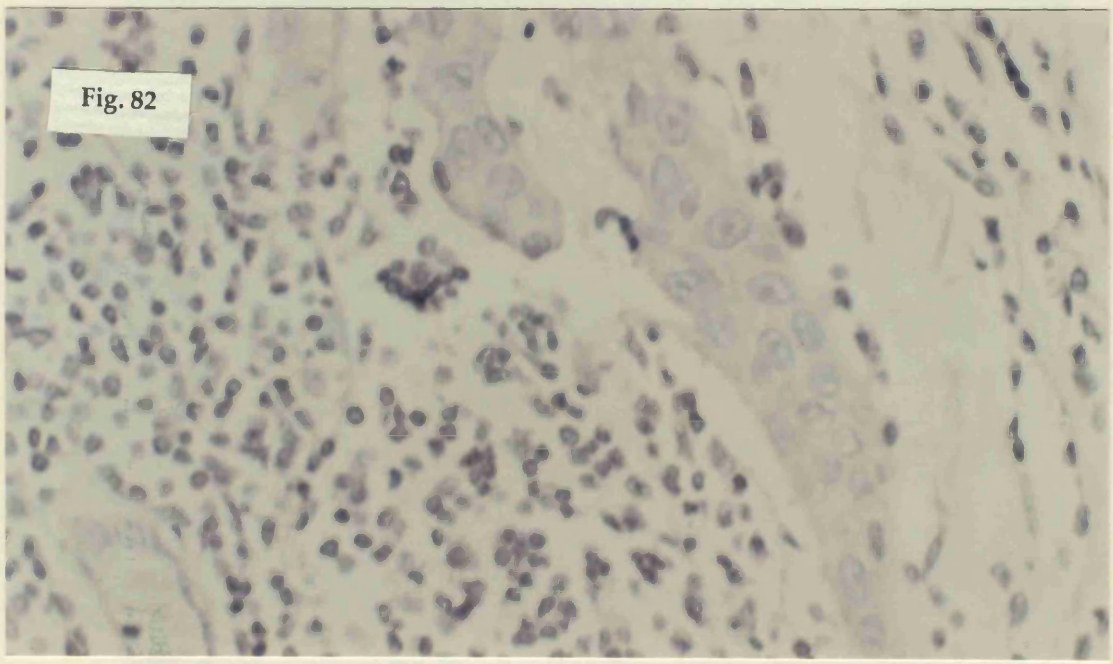
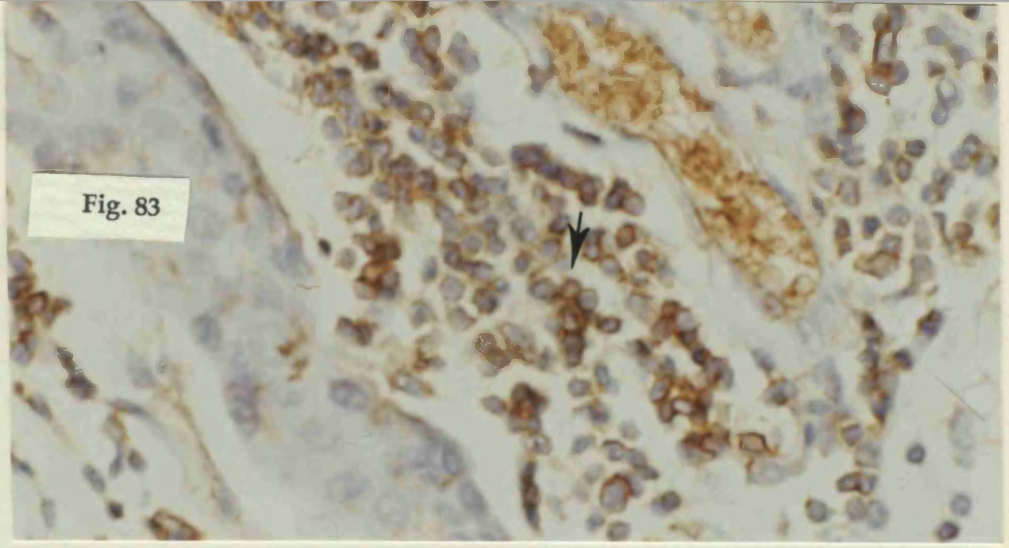
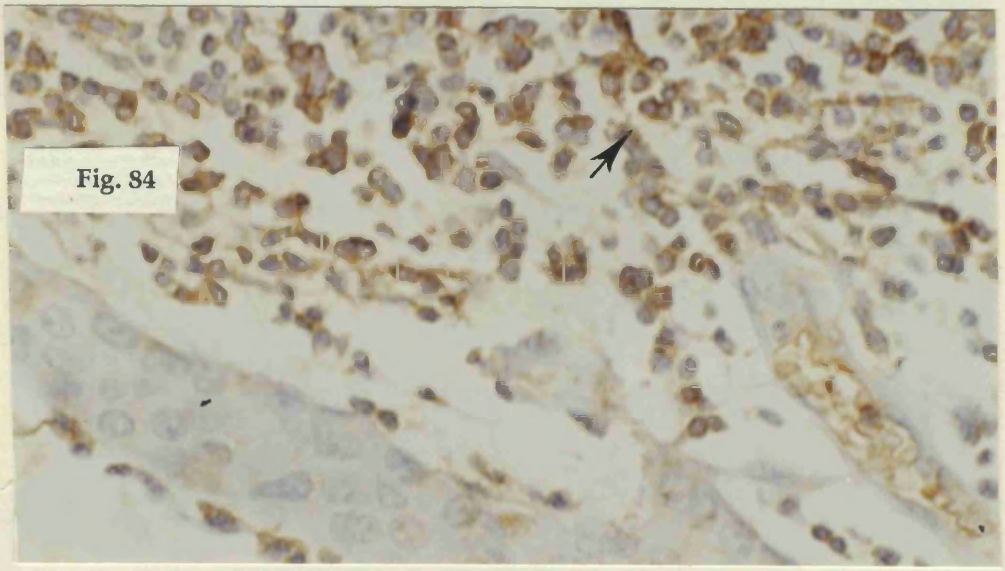


Fig. 82 Same case as in Fig. 81. Negative control.
Both cancer cells and lymphocytes are completely -ve.
X 40





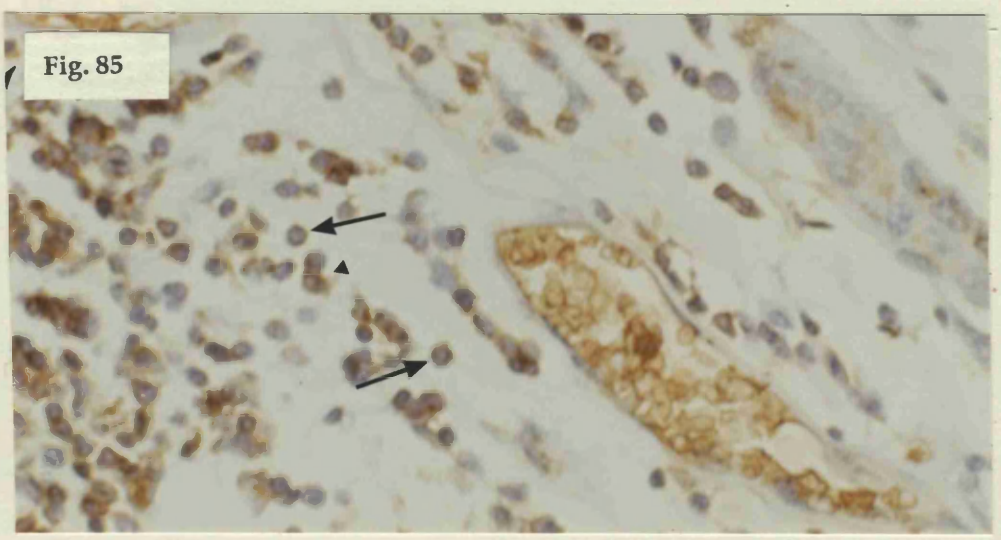


Fig. 83 Same case as in Fig. 81, stained with MLI.

Fig. 84 Same case as in Fig. 81, stained with MLII.

Fig. 85 Same case as in Fig. 81, stained with MLIII.

Malignant cells are -ve . Lymphocytes are +ve (arrowed). RBCs are also +ve.
X 40

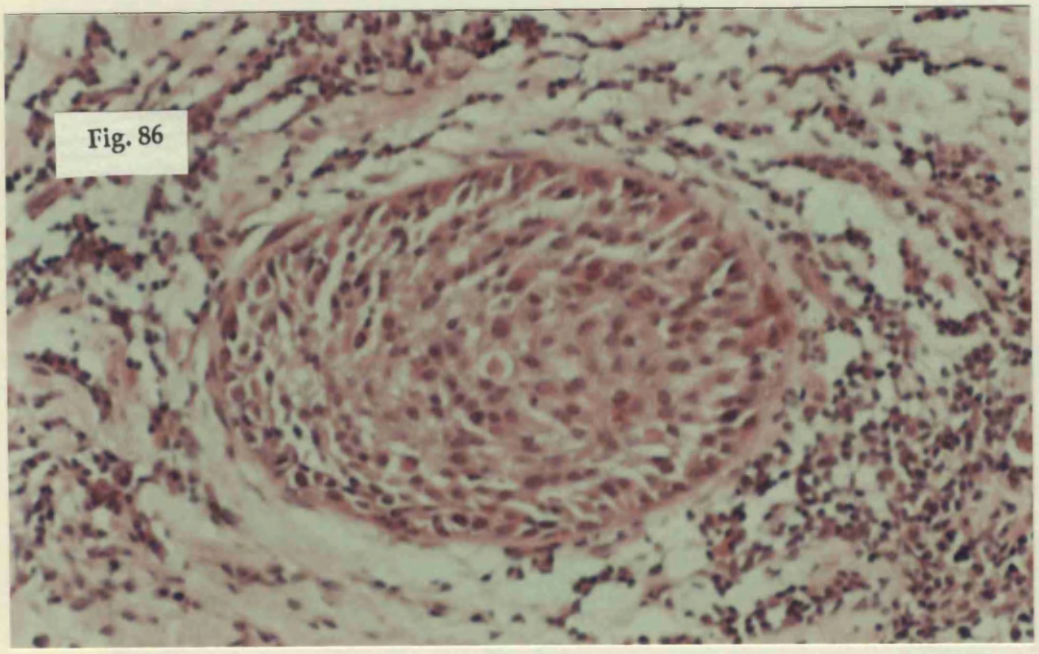


Fig. 86 Section of primary breast carcinoma stained with H & E. The section was of moderately-differentiated ductal carcinoma.
X 20

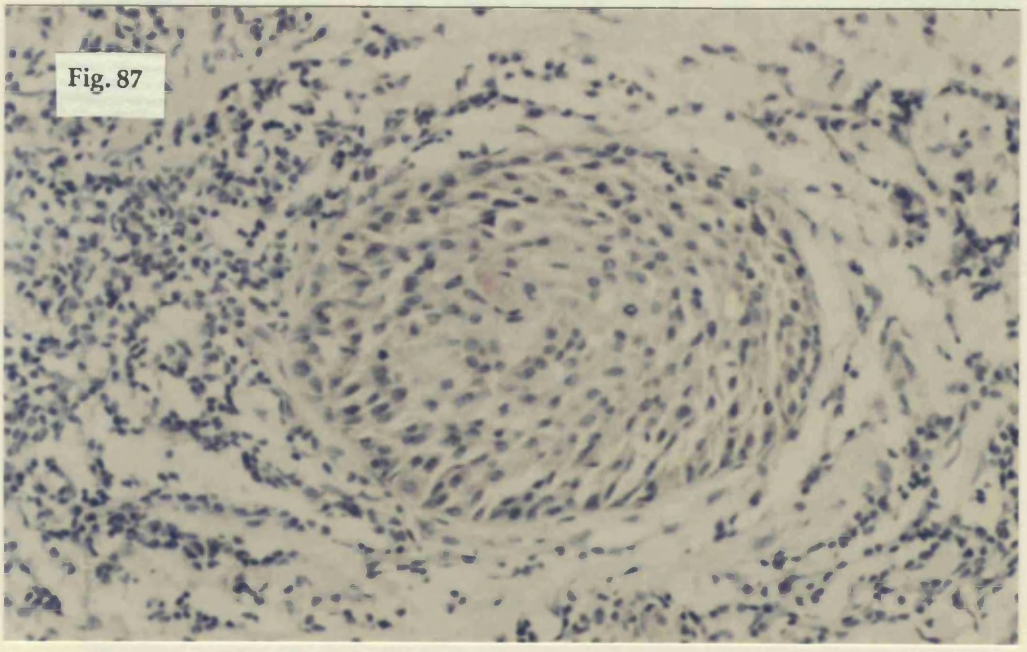
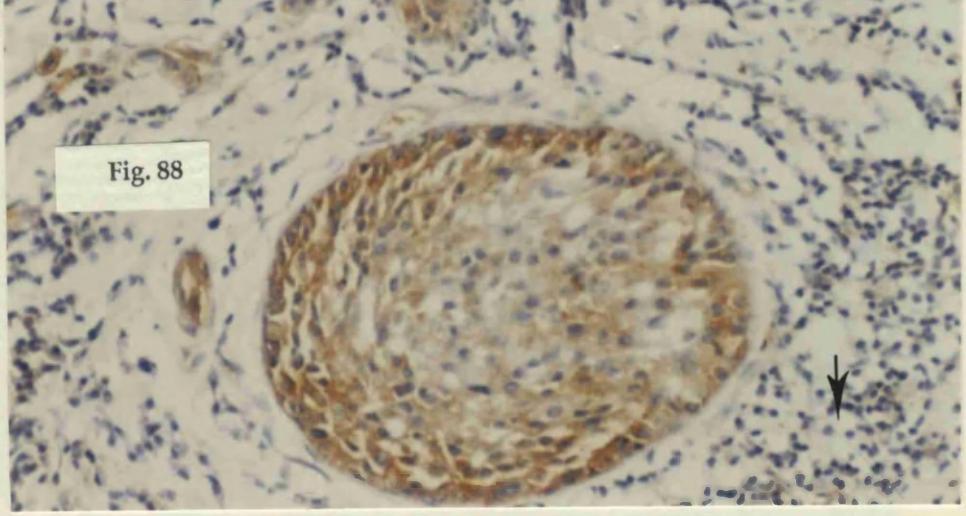
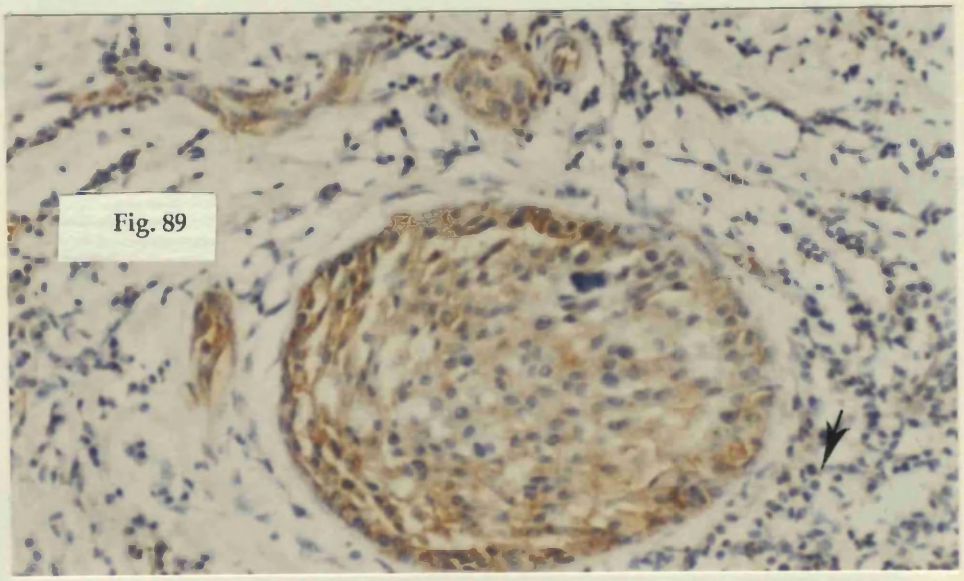


Fig. 87 Same case as in Fig. 86. Negative control.
No staining is present.
X 20





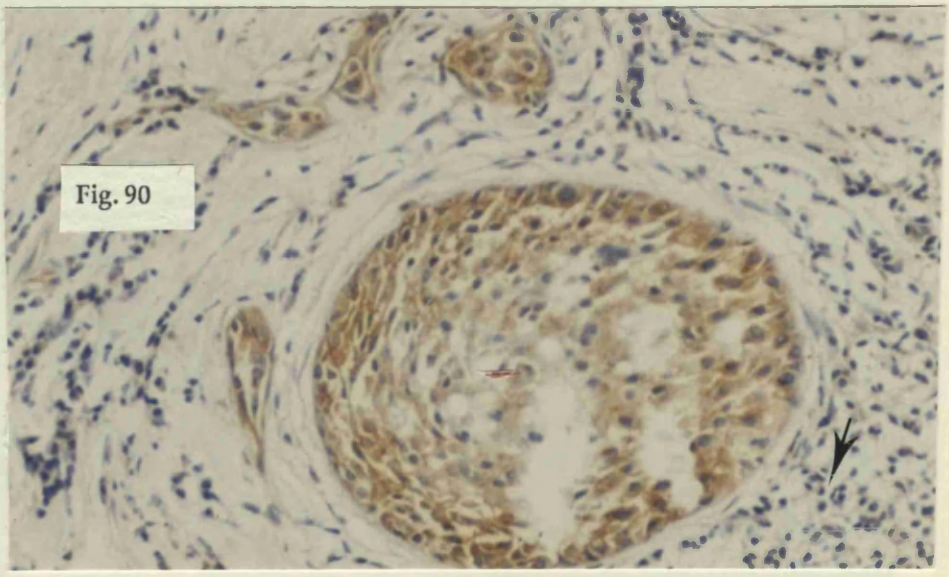


Fig. 88 Same case as in Fig. 86, stained with MLI.

Fig. 89 Same case as in Fig. 86, stained with MLII.

Fig. 90 Same case as in Fig. 86, stained with MLII.

Malignant cells are +ve, lymphocytes (arrowed) are -ve.

X 20

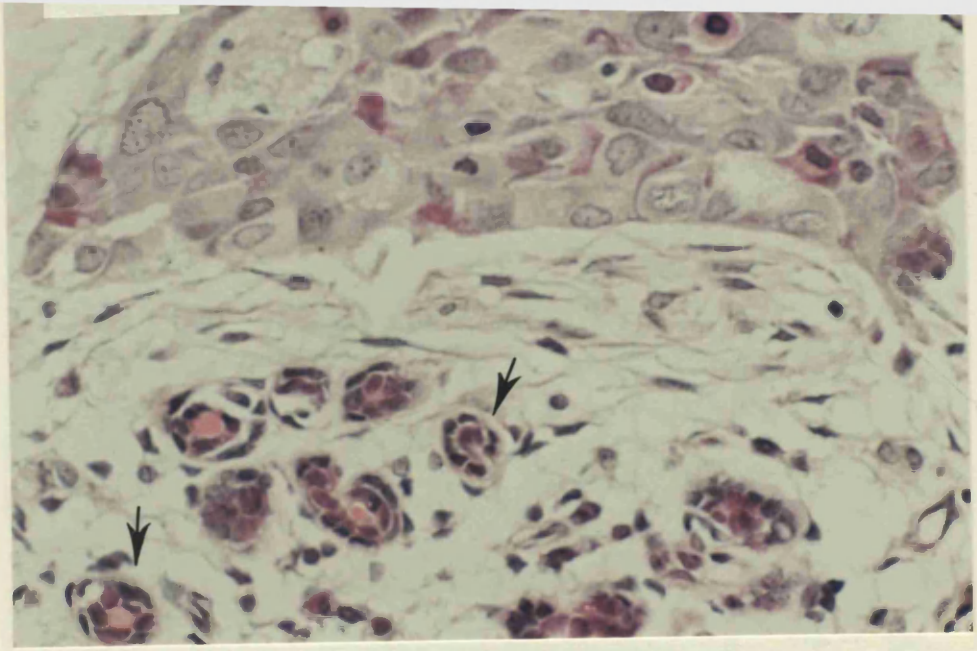


Fig. 91 Section of primary breast carcinoma stained with H & E, showing benign components (arrowed) in addition to cancer cells. Tumour was composed of sheets and strands of large polygonal cells.
X 40

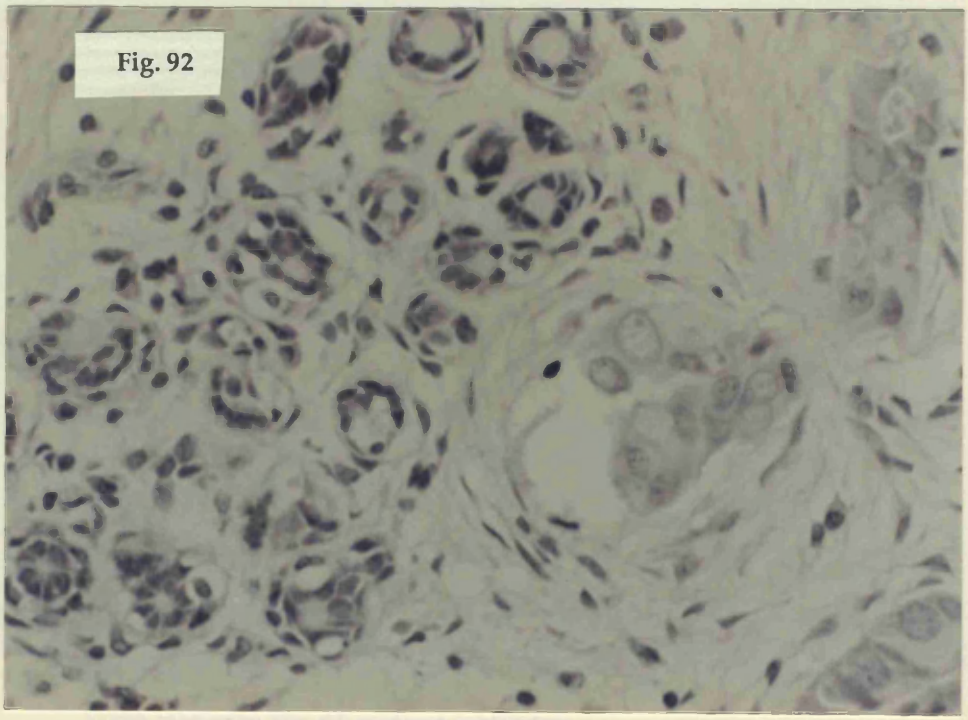
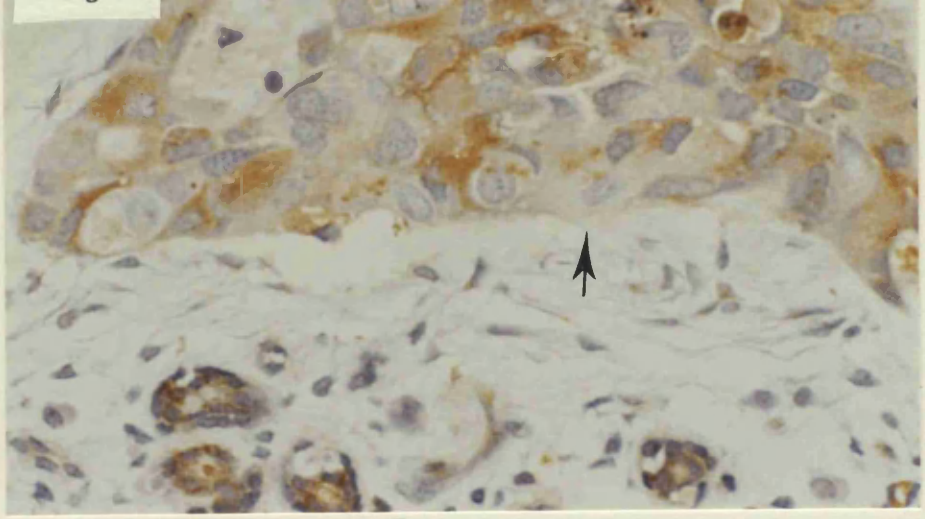
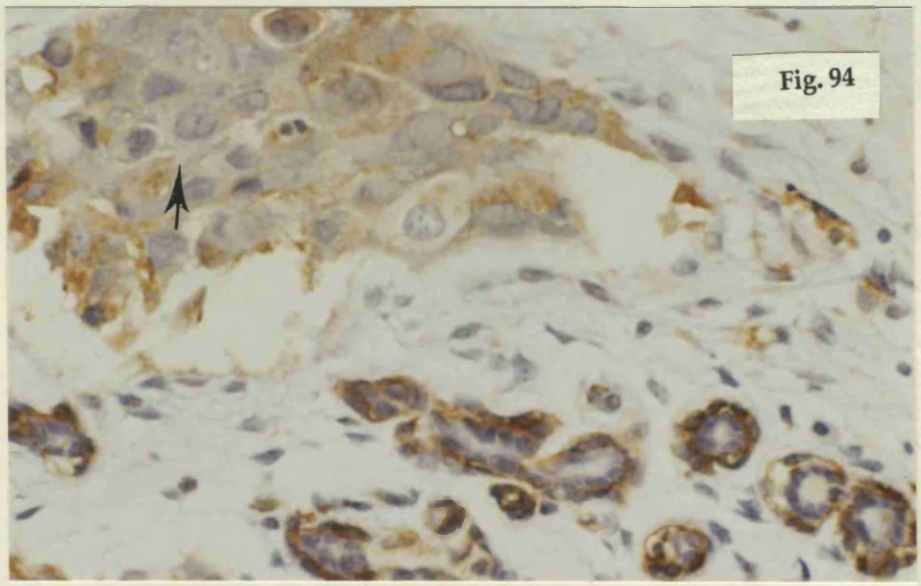


Fig. 92 Same case as in Fig. 91. Negative control.
Benign and malignant cells are negative.
X 40





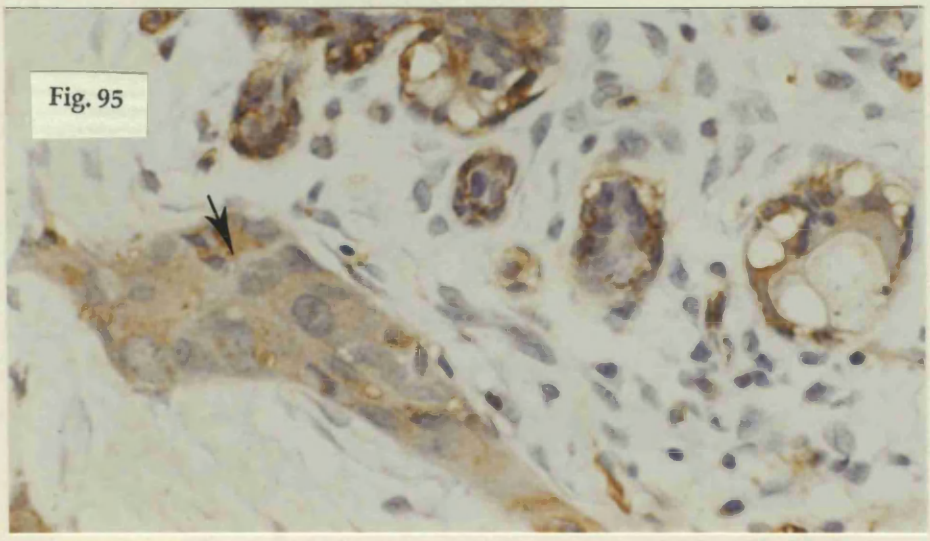


Fig. 93 Same case as in Fig. 91, stained with MLI.

Fig. 94 Same case as in Fig. 91, stained with MLII.

Fig. 95 Same case as in Fig. 91, stained with MLIII.

The malignant cells are showing heterogeneity in their reactivity to MLI. Some are -ve (arrowed). Benign components are showing faint cytoplasmic positivity with strong luminal positivity.

X 40

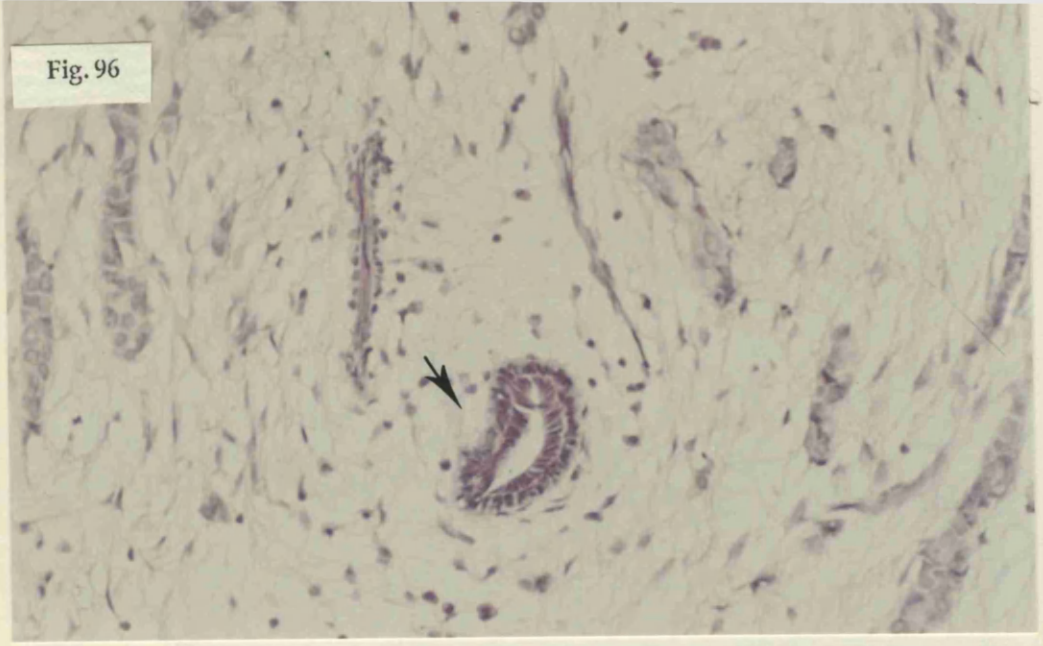


Fig. 96 Section of primary breast carcinoma stained with H & E, showing benign components (arrowed) in addition to cancer cells. The case was well-moderately differentiated ductal carcinoma.
X 20

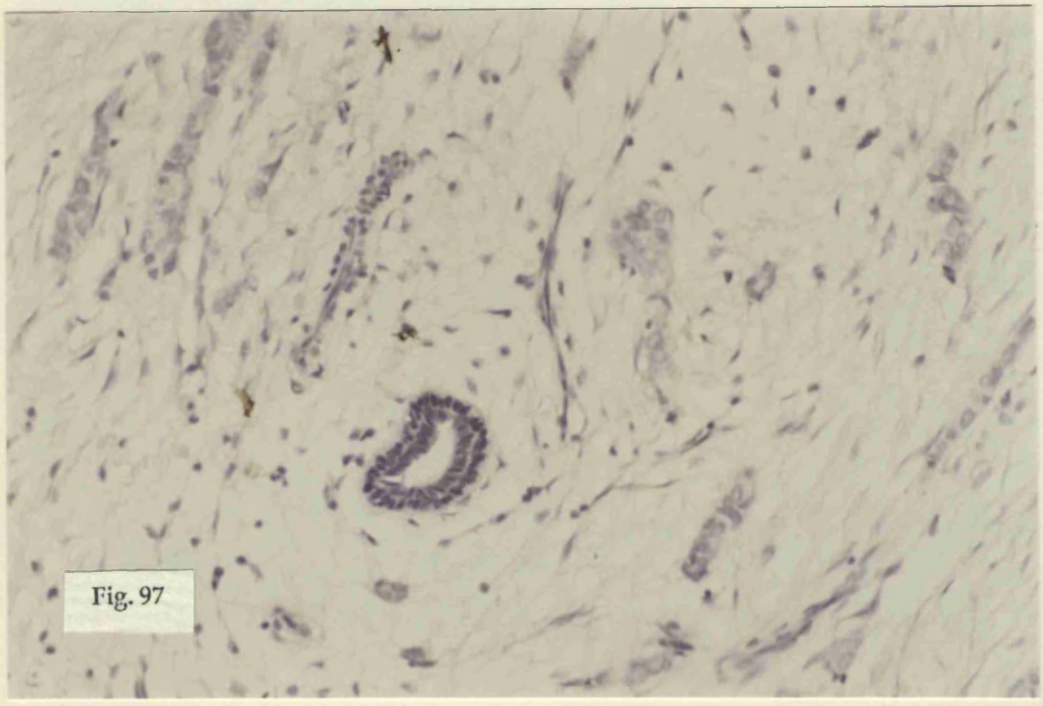
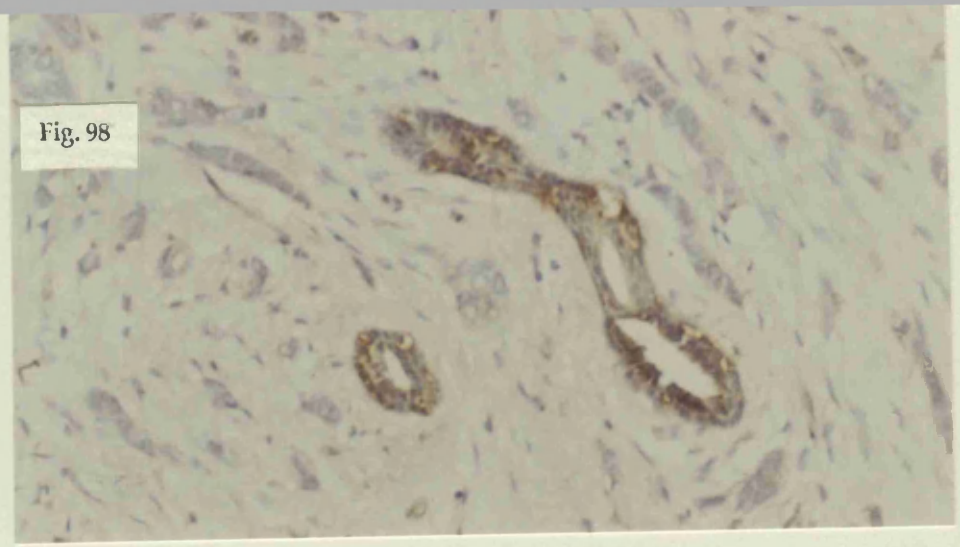
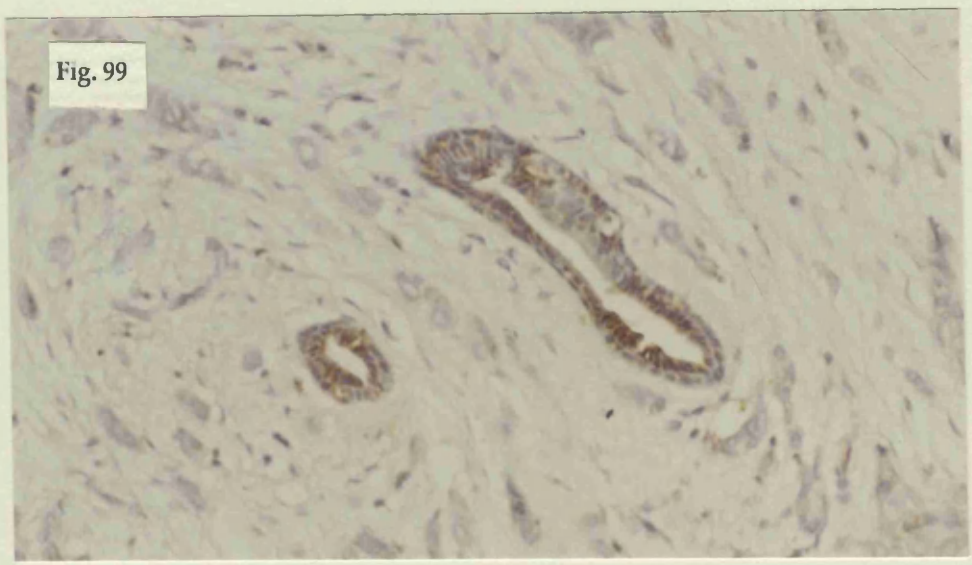


Fig. 97 Same case as in Fig. 96. Negative control. Malignant and benign cells are negative.
X 20





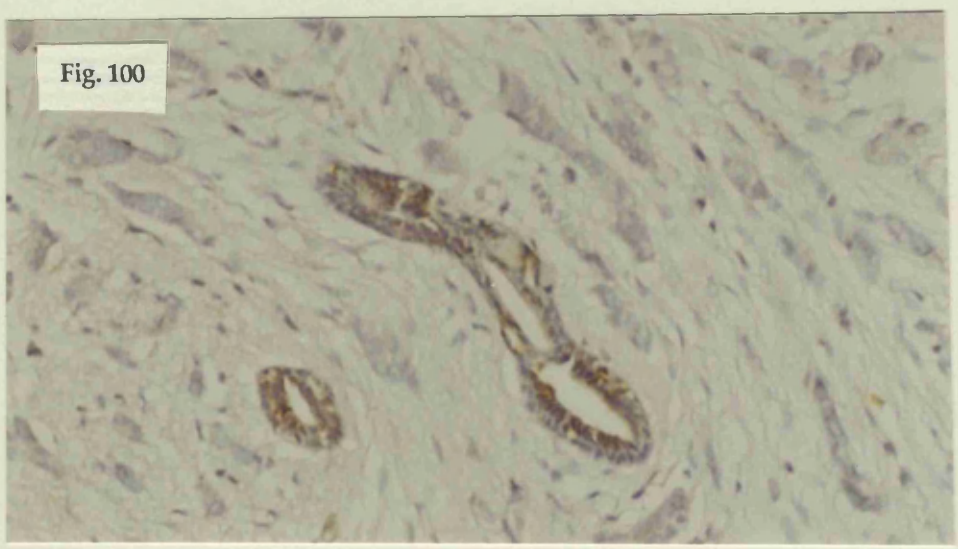


Fig. 98 Same case as in Fig. 96, stained with MLI.
Fig. 99 Same case as in Fig. 96, stained with MLII.
Fig. 100 Same case as in Fig. 96, stained with MLII.
Malignant cells are negative while benign cells are +ve.
X 20

The most important aim of this study was to find out if the binding of mistletoe lectins correlated with clinical behaviour of these cancers. The next section describes the correlation of Mistletoe lectins binding with clinical behaviour of breast cancers.

4. STATISTICAL ANALYSES

A total of 234 cases of primary breast cancer were stained for MLI, MLII and with MLIII. These represent all 193 primary breast cancer cases, where tissues were available, presented at the Bland-Sutton Institute, Middlesex Hospital between 1978 and 1980 plus 41 miscellneous primary breast cancer cases presented between 1981 and 1985. 193 cases were all the cases of 1978, 1979 & 1980 where their primary breast cancer tissue was found (in some cases the primary cancer tissue was missing, these cases were not included).

The clinical progress of these patients during follow-up for a minimum period of 9 years and maximum period of 11 years was found by means of patient's notes, the Middlesex Hospital Cancer Registry, and Cancer Death Registry. When these attempts failed to determine the follow-up of some patients, letters were written to their general practitioners to seek information concerning recurrences and survival.

The staining (positive, negative or unknown) was correlated with the patient's blood group (A, B, AB, O, unknown), histology type (ductal, lobular, polygonal, mucinous, squamous, colloidal, comedo and others), differentiation (poor, moderate and well), of the tumours, lymph node (LN) status, survival and disease free interval (DFI).

All 234 cases were considered suitable for correlating the staining with LN status and with primary tumour size. However, inclusion of the 41 miscellaneous cases in survival analysis may introduce bias as there were obviously other similar cases on whom no clinical information at all is available. For this reason, the survival and the DFI analysis were carried out on the less biased 193 cases.

Log-rank analysis and life-tables were prepared by the Kaplan-Meier method using a programme written by David Bradley, Queen's University, Belfast, for the department of Radiotherapy, Middlesex Hopspital.

Mantel-Cox P-value, to see if the observed differences, if any, between the different groups could be just a chance, was used. The Mantel-Cox test gives greater weight to late observations and is sensitive to events which occur when few patients on the study remains alive (Mantel, 1966).

The statistical analyses were kindly performed by Teresa Young of the Department of Radiotherapy and Oncology at the Middlesex Hospital.

4.1. correlation of staining with patient's menopausal status

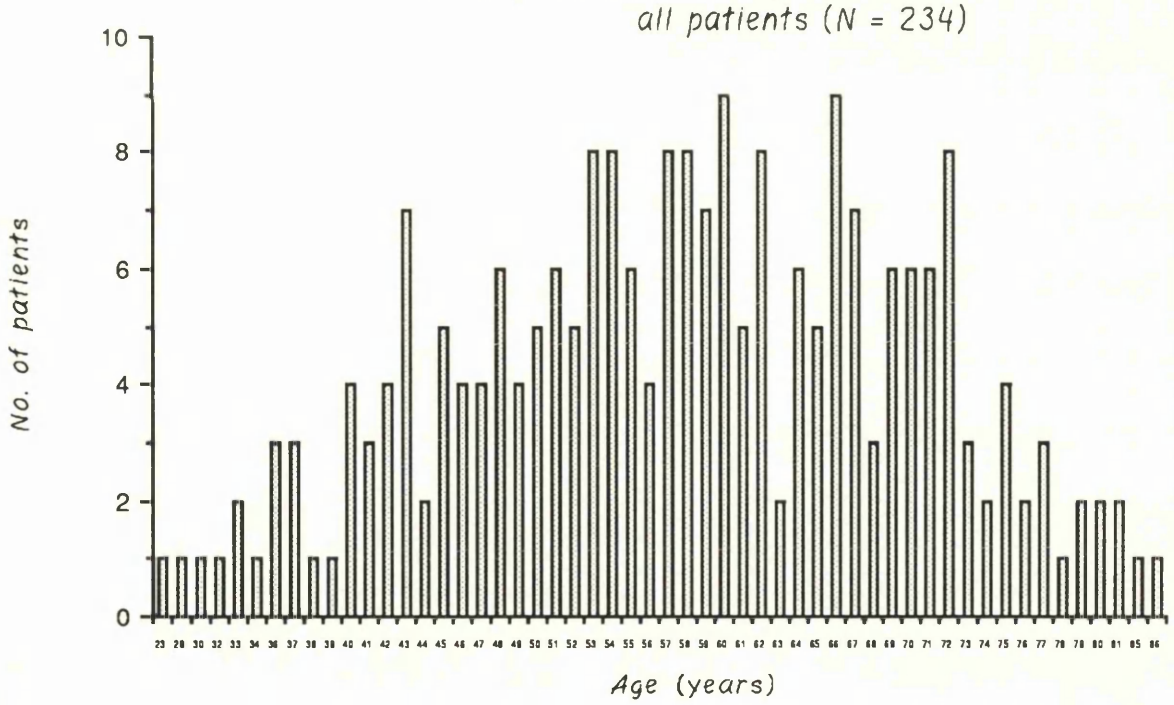
4.1.1. Menopausal status of patients at diagnosis

The age distribution of the 234 cases was analysed (Histogram, 1).

The minimum age in the series was 23 and the maximum was 86.

The mean age value was = 57.7.

Histogram (1) showing age distribution of all patients (N = 234)



Menopausal status was initially recorded from notes, but the information was lacking for many patients; therefore menopausal status was assumed from age. Women of 50 years and younger were assumed to be premenopausal (presumed premenopausal), those over 50 years postmenopausal (presumed postmenopausal). There were 65 patients in presumed premenopausal age group while there were 165 patients in the presumed postmenopausal group. No information on the age of 4 patients was available.

4.1.2. Menopausal status/ MLI staining

Information about age of the patients and their tissue staining results using MLI was available on 217 cases (Table; 6).

Table; 6

Staining results of MLI to tissues of patients with different menopausal status

Status	MLI staining		
	+VE	-VE	Total
Postmenopausal	98	60	158
Premenopausal	32	27	59
Total	130	87	217

4.1.3. Menopausal status/ MLII staining

Information on age of the patients and their tissue staining results using MLII was available on 218 cases (Table; 7).

Table; 7

Staining results of MLII to tissues of patients with different menopausal status

Status	MLII staining		
	+VE	-VE	Total
Postmenopausal	100	59	159
Premenopausal	35	24	59
Total	135	83	218

4.1.4. Menopausal status/ MLIII staining

Information about age of the patients and their tissue staining results using MLIII was also available on 218 cases (Table, 8).

Table; 8

Staining results of MLIII to tissues of patients of different menopausal status

Status	MLIII staining		
	+VE	-VE	Total
Postmenopausal	98	61	159
Premenopausal	34	25	59
Total	132	86	218

4.2. correlation of staining with patient's blood group

4.2.1. Blood groups

The first question asked was whether the stainers for MLI and/or MLII and/or MLIII are of a particular blood group (A, B, AB, O)?

information on patient's blood groups were obtained from patient's notes. Out of 234 cases stained for the 3 lectins, information on blood groups and staining results were available on 68 cases for MLI and on 69 cases for MLII & MLIII.

4.2.2. Blood group/ MLI staining

No correlation between patient's blood group and staining for MLI was found ($P < 0.3593$). As outlined in the following table (table; 9), the stainers are of different blood groups:

Table; 9

Staining results of MLI to tissues of patients with different blood groups

Blood group	MLI staining		
	+ve	-ve	Total
O	17	11	28
A	22	10	32
B	3	3	6
AB	0	2	2
Total	42	26	68

Patients with blood group AB were only 2, too few to draw any conclusion from their staining results.

4.2.3. Blood group/ MLII staining

Stainers for MLII were also from different blood groups. No correlation between patient's blood group and staining (P < 0.380) was found (Table, 10)

Table; 10

Staining results of MLII to tissues of patients with different blood groups

Blood group	MLII staining		
	+ve	-ve	Total
O	16	12	28
A	22	10	32
B	4	3	7
AB	0	2	2
Total	42	27	69

4.2.4. Blood group/ MLIII staining

As with MLI and MLII, no correlation was found between patient's blood group and staining for MLIII (P < 0.380) (Table, 11)

Table; 11

Staining results of MLIII to tissues of patients with different blood groups

Blood group	MLIII staining		
	+ve	-ve	Total
O	16	12	28
A	22	10	32
B	4	3	7
AB	0	2	2
Total	42	27	69

4.3. correlation of staining with primary tumour size

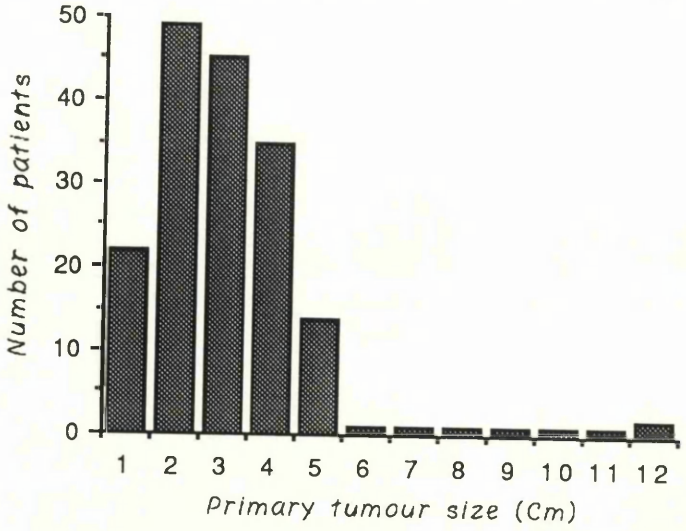
4.3.1. Primary tumour size

The primary tumour size of the patients was taken as the maximum dimension (rounded to the nearest cm). Information on primary tumour size of patients was obtained from reports of the pathologist who dealt with the primary tumour. No information on primary tumour size of 61 patients as these patients were either not subjected to lumpectomy or mastectomy or no record of the size of their primary tumour was found. Primary tumour size of 173 patients was available. The minimum primary tumour size was = 1 cm and the maximum was 12 cm. The mean value of the primary tumour size of the 173 patients was = 3.11cm. There were 22 patients with primary tumour size of 1 cm.

As illustrated in the following histogram, 49 patients had a primary tumour size of 2 cm. 45 patients had 3 cm size primary tumour, 35 patients had 4 cm and 14 patients had 5 cm.

For correlation with staining, patient's primary tumour size was divided into 3 groups: less or equal to 2 cm, 2-5 cm, and >5cm.

Histogram (2) Primary tumour size of patients (N=173)



4.3.2. Primary tumour size/ MLI staining

When the staining for MLI correlated with primary tumour size of patients , no significant correlation was found (P < 0.883). The following table shows that this analysis was done on 165 cases where primary tumour size and staining results using MLI were available (staining = unknown was excluded).

Table; 12

Staining results of MLI to tissues of patients with different tumour sizes

MLI staining	1°TUMOUR SIZE			
	<2cm	2-5cm	>5	TOTAL
+ve	45	54	5	104
-ve	24	34	3	61
TOTAL	69	88	8	165

4.3.3. Primary tumour size/ MLII staining

No significant correlation was found between patient's primary tumour size and staining for MLII (P < 0.9815). information on primary tumour size and staining results using MLII were available on 166 cases (Table, 13)

Table; 13

Staining results of MLII to tissues of patients with different tumour sizes

MLII staining	1°TUMOUR SIZE			
	<2cm	2-5cm	>5	TOTAL
+ve	45	57	5	107
-ve	24	32	3	59
TOTAL	69	89	8	166

4.3.4. Primary tumour size/ MLIII staining

Information on primary tumour size and staining results using MLIII were available on 166 cases. No significant correlation was found between patient's primary tumour size and MLIII staining ($P < 0.9930$) (the following table, 14)

Table; 14

Staining results of MLIII to tissues of patients with different tumour sizes

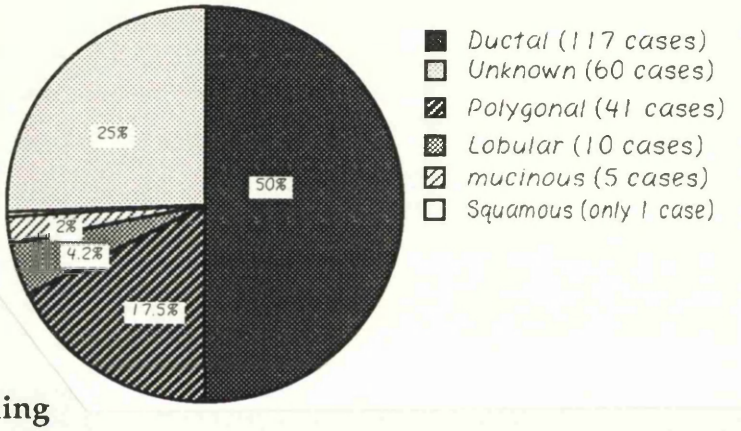
MLIII staining	1°TUMOUR SIZE			
	<2cm	2-5cm	>5	TOTAL
+ve	44	56	5	105
-ve	25	33	3	61
TOTAL	69	89	8	166

4.4. correlation of staining with histological type

4.4.1. Histology

The histological types of primary breast cancer cases were obtained from the reports of the histopathologists who diagnosed the cancer cases at that time. The distribution of the different histological types used in the study is presented below in Pie diagram; 1:

A pie diagram showing histological types of the 234 primary breast cancer cases



4.4.2. Histology/ MLI staining

No correlation was found between histological type and staining for MLI ($P < 0.6110$). The results are shown in the following table (Table, 15):

Table; 15

Staining results of MLI to tissues of patients with different tumour histology

Histology	MLI staining +ve	-ve	unknown	Total
Ductal	64	43	10	117
Polygonal	22	17	2	41
Lobular	8	2	0	10
Mucinous	2	3	0	5
squamous	1	0	0	1
Unknown	33	22	5	60
Total	130	87	17	234

4.4.3. Histology/ MLII staining

No correlation was found between histological type and staining for MLII ($P < 0.6658$). The results are shown in the following table (Table, 16):

Table; 16

Staining results of MLII to tissues of patients with different tumour histology

Histology	MLII staining			Total
	+ve	-ve	unknown	
Ductal	69	39	9	117
Polygonal	22	17	2	41
Lobular	7	3	0	10
mucinous	2	3	0	5
squamous	1	0	0	1
Unknown	34	21	5	60
Total	135	83	16	234

4.4.4. Histology/ MLIII staining

No correlation was found between histology type and staining for MLIII ($P < 0.6774$). The results are shown in the following table:

Table, 17

Staining results of MLIII to tissues of patients with different tumour histology

Histology	MLIII staining			Total
	+ve	-ve	unknown	
Ductal	68	40	9	117
Polygonal	21	18	2	41
Lobular	6	4	0	10
mucinous	2	3	0	5
squamous	1	0	0	1
Unknown	34	21	5	60
Total	132	86	16	234

4.5. correlation of staining with tumour grades (differentiation)

4.5.1. Tumour grade (differentiation)

Tumour grade of cancer cases as either grade I (well differentiated), grade II (moderately differentiated) or grade III (poorly differentiated) was obtained from histopathological reports.

4.5.2. Tumour grade/ MLI staining

No correlation was found between the degree of differentiation of tumours and staining for MLI ($P < 0.7832$). The results are shown in the following table Table, 18), where G = Grade:

Table; 18

Staining results of MLI to tissues of patients with different tumour grades

Grade	MLI staining			Total
	+ve	-ve	unknown	
G III	56	36	5	97
G II	15	14	3	32
G I	5	2	0	7
Unknown	54	35	9	98
Total	130	87	17	234

4.5.3. Tumour grade/ MLII staining

No correlation was found between the degree of differentiation of tumours and staining for MLII ($P < 0.7617$). The results are shown in the following table (Table, 19):

Table; 19

Staining results of MLII to tissues of patients with different tumour grades

Grade	MLII staining			Total
	+ve	-ve	unknown	
G III	61	31	5	97
G II	17	12	3	32
G I	5	2	0	7
Unknown	52	38	8	98
Total	135	83	16	234

4.5.4. Tumour grade/ MLIII staining

No correlation was found between the degree of differentiation of tumours and staining for MLIII ($P < 0.8066$). The results are shown in the following table (Table, 20):

Table; 20

Staining results of MLIII to tissues of patients with different tumour grades

Grade	MLIII staining			Total
	+ve	-ve	unknown	
G III	59	33	5	97
G II	17	12	3	32
G I	5	2	0	7
Unknown	51	39	8	98
Total	132	86	16	234

4.6. correlation of staining with lymph node (LN) status

4.6.1. LN status

LN status was based on histological evidence of axillary LN involvement, ie, the LNs were removed at sugery and the presence of metastastatic tumour cells was confirmed by histopathological report. Only then LN status was considered positive. Where any number of LNs were sampled and histological investigation did not confirm the presence of metastatic cells, the LN status was considered negative. In cases where LNs were not removed at surgery or not sampled, the LN status was recorded as unknown. Out of 234 patients, information on LN status was available on 151 as 83 cases were unknown. Of the 151 cases, 83 cases were LN+ve and 68 cases were LN-ve. .

4.6.2. LN status/ MLI staining

Information on LN status and staining results using MLI was available on 139 case. The results are presented in the following table (Table; 21).

Table; 21

Staining results of MLI to tissues of patients with different LN status

MLI staining	LN Status		
	+ve	-ve	Total
+ve	48	42	90
-ve	28	21	49
Total	76	63	139

No significant correlation was found between MLI staining and LN status ($P < 0.8005$)

4.6.3. LN status/ MLII staining

The following table shows that information on LN status and staining for MLII was available on 140 cases, of which 77 cases were LN+ , 52 of them stained positively for MLII, and out of 63 LN- cases, 39 were positive for MLII. The rest were -ve.

Table; 21

Staining results of MLII to tissues of patients with different LN status

MLII staining	LN Status		
	+ve	-ve	Total
+ve	52	39	91
-ve	25	24	49
Total	77	63	140

No significant correlation was found between MLII staining and LN status ($P < 0.6055$)

4.6.4. LN status/ MLIII staining

The following table shows that out of 77 LN+ cases 51 stained positively for MLIII, and out of 63 LN- cases, 39 were positive for MLIII. The rest were -ve.

Table; 23

Staining results of MLIII to tissues of patients with different LN status

MLIII staining	LN Status		
	+ve	-ve	Total
+ve	51	39	90
-ve	26	24	50
Total	77	63	140

No significant correlation was found between MLIII staining and LN status ($P < 0.7229$)

4.7. correlation with survival

4.7.1. Survival

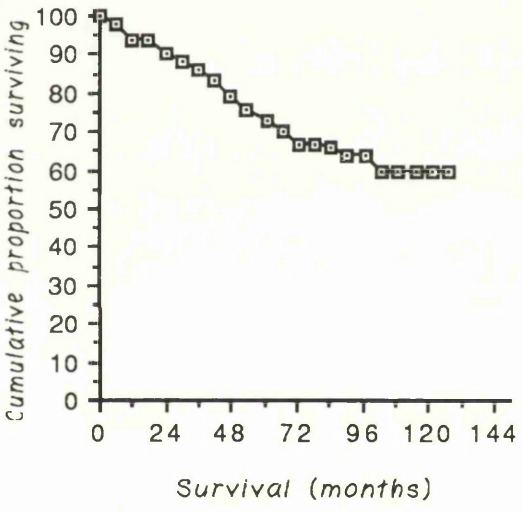
Survival is the time between surgery (ie, histological confirmation of malignant disorder) and death. Times were recorded in months. This information were obtained from patient's notes.

When the patients were lost to follow up immediately after surgery, their survivals were calculated as one day after the date of operation or biopsy. When the patients were lost to follow-up sometime after surgery, their survivals were calculated as one day after the date they were last seen (Friedman et al, 1983). Including these patients was essential to reduce bias of statistical analysis. Out of the 193 cases analysed for survival and have been followed up for a minimum period of 9 yrs and a maximum

period of 11 yrs, information on survival was lost on 105 cases. Out of the remaining cases; 45 died of breast cancer, 1 died not because of breast cancer and 42 are still alive.

The following Life-table (Fig. 101) shows survival of the 193 patients.

Fig. 101 Survival of all patients (N=193)



At 5 years, the rate of survival of all patients is 74% and at 10 yrs is 60% (table; 24).

Table; 24

Survival of all patients (N=193)

at 5 yrs	at 10 yrs
74%	60%

4.7.2. Survival of patients according to presumed menopausal status

The age distribution of the 193 cases was analysed.

The minimum age in the series was 23 and the maximum was 86.

The mean age value was = 58.3.

51 patients in the premenopausal group and 136 patients in the postmenopausal group. No information on the age of 4 patients was available. When the 193 cases were divided into two age groups: premenopausal (presumed) and postmenopausal (presumed), the number of patients in the younger group was 51 of whom 10 died of breast cancer (died BC), 9 still alive, and 32 were lost to follow-up. The number of patients in the older group was 142 of whom 25 died BC, 1 died not because of breast cancer (died NBC), 33 still alive and 83 were lost to follow-up (table; 25)

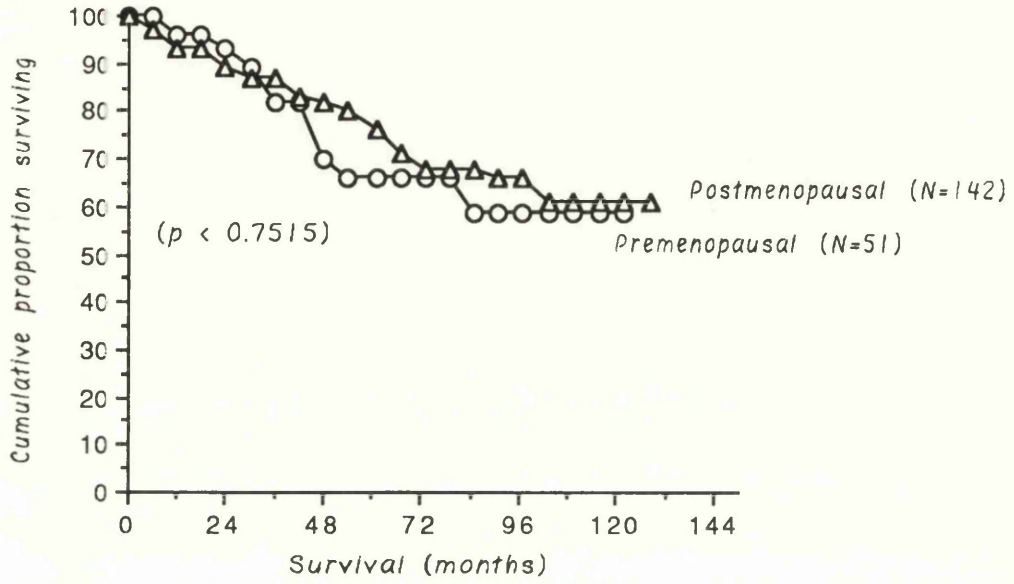
Table; 25

Survival of patients according to their menopausal status

	Total	Died BC	Died NBC	Lost	Alive
Premenopausal	51	10	0	32	9
Postmenopausal	142	25	1	83	33
Totals	193	35	1	115	42

The following Life-table (Fig. 102) shows the rate of surviving in the premenopausal group and in postmenopausal group

Fig. 102 Survival of the 193 patients divided into 2 age groups: Postmenopausal and premenopausal



The following table (table; 26) shows the survival rates of the different groups at both 5- and 10-year intervals:

Table; 26

Survival rates of patients according to their menopausal status

status	Survival		
	5 yrs	10 yrs	p-value
Premenopausal	65%	58%	< 0.7515
Postmenopausal	68%	61%	

The above presented data suggest that the probability of surviving in the postmenopausal patients is higher than those of the premenopausal patients, although the difference statistically was not significant ($p < 0.7515$).

4.7.3. Survival/Primary tumour size

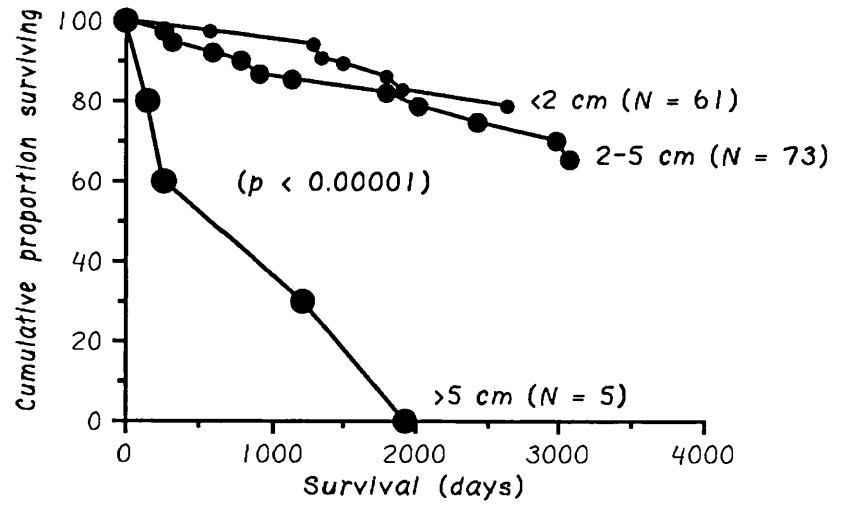
information on primary tumour size and survival was available on 139 cases. The results are presented in the following table (table; 27).

Table; 27

Survival of patients according to their 1° tumour size

1° size	Total	Died BC	Died NBC	Alive	lost
<2cm	61	7	0	23	31
2-5cm	73	10	1	17	45
>5cm	5	4	0	1	0
Totals	139	21	1	77	40

Fig. 103 Survival/ patients are divided according to their primary tumour size (N = 139)



From the above life-table (Fig. 103), survival rates of patients according to their tumour size, at 5- and 10-year intervals, are presented in the following table (table; 28).

Table; 28

Survival rates of patients according to their 1° tumour size

1° size	Survival 5-year	10-year	P-value
<2cm	88%	72%*	
2-5cm	82%	52%*	< 0.00001
>5cm	20%	0	

* by extrapolation

Only 5 cases were of tumour size of larger than 5cm in diameter; non of them survived longer than 5 yrs. So the correlation with the other 2 tumour sizes is not possible. However, the survival table suggests an inverse relationship between primary tumour size and survival rate.

4.7.4. Primary tumour size/ survival according to menopausal status

The results of correlation are presented in table 29.

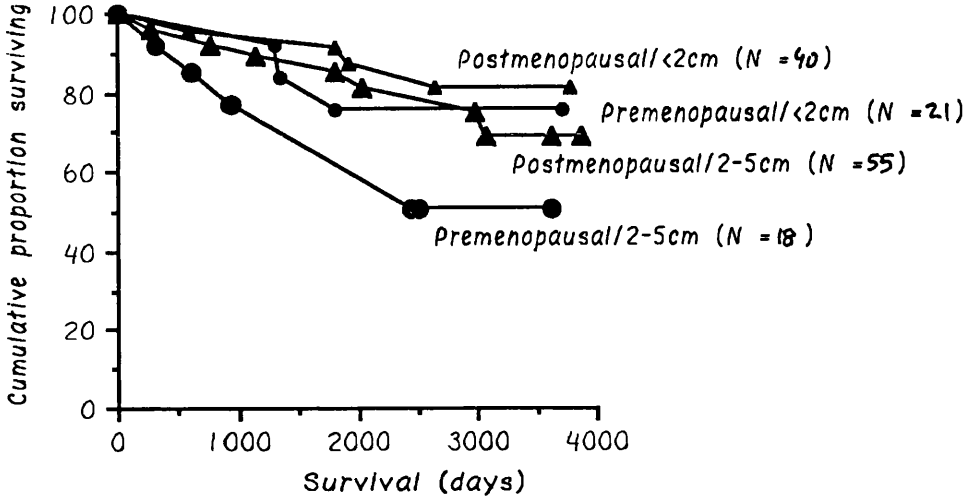
Table; 29

Survival of patients according to their menopausal status and their 1° tumour size

status	Total	Died BC	Died NBC	Alive	Lost
postmenopausal/<2	40	4	0	16	20
postmenopausal/2-5	55	6	1	15	33
Postmenopausal/>5	5	4	0	0	1
premenopausal/<2	21	3	0	7	11
premenopausal/2-5	18	4	0	2	12
Totals	139	21	1	40	77

The following life table (Fig. 104) shows that postmenopausal patients do better than premenopausal when they have a large tumour size.

Fig. 104 Survival/ patients are divided according to their tumour size and menopausal status



Table; 30

Survival rates of patients according to their menopausal status and their 1° tumour size

Status	Survival 5-yr	10-yr
postmenopausal/<2cm	88%	82%
postmenopausal/2-5cm	83%	68%
premenopausal/<2cm	78%	76%
premenopausal/2-5cm	60%	51%

4.7.5. Tumour grade correlated with survival

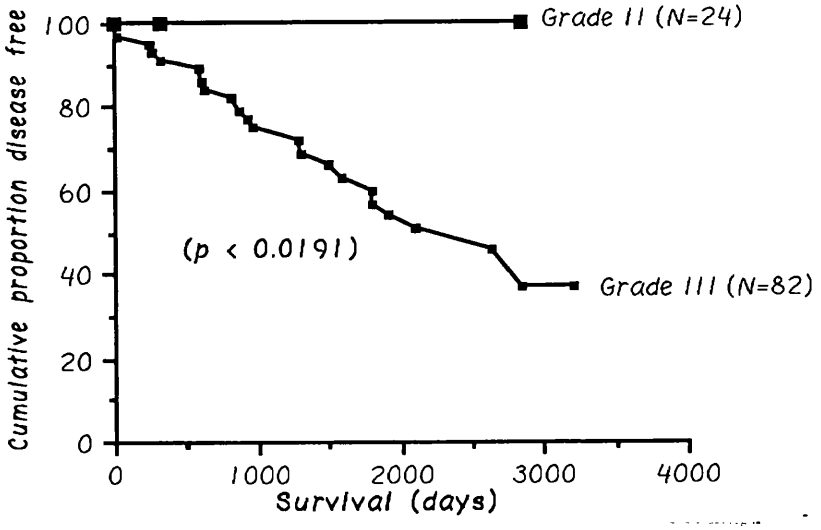
information on survival and tumour size were available on 113 cases. The results are presented in the following table (table; 31), where G = Grade:

Table; 31

Survival of patients according to their tumour grade

Grade	Total	Died BC	Died NBC	Alive	lost
G III	82	20	0	11	51
G II	24	0	0	18	6
G I	7	1	0	0	6
Totals	113	21	0	17	75

Fig. 105 Survival/ patients are divided according to their tumour grade



From the above life-table Fig. 105), survival of patients according to their tumour grade, at 5- and 10-year intervals are presented in the following table (table; 32).

Table; 32

Survival rates of patients according to their tumour grade

Grade	Survival		
	5-year	10-year	P-value
G II	100	100*	< 0.0191
G III	57%	37%*	

* by extrapolation

Only 7 patients had well differentiated tumours, while 82 patients had poorly differentiated tumours. Although correlation of survival of patients of high grade tumour with those with low grade is not possible in this study, correlation with grade II tumours suggest that patients with higher grade do much worse than those with lower grade.

4.7.6. Tumour grade/ survival according to menopausal status

The results are presented in the following table:

Table; 33

Survival of patients according to their menopausal status and their tumour grade

	Total	Died BC	Died NBC	Alive	Lost
postmenopausal/GI	4	0	0	0	4
postmenopausal/GII	20	0	0	5	15
Postmenopausal/GIII	52	13	0	10	29
premenopausal/GI	3	1	0	1	22
premenopausal/GII	4	0	0	1	3
Premenopausal/GIII	30	7	0	0	2
Totals	113	21	0	17	75

The following life-table (Fig. 106) shows that patients with grade III tumours do worse if they are premenopausal.

Fig. 106 Survival/Patients with poorly differentiated tumours divided into 2 groups according to their menopausal status

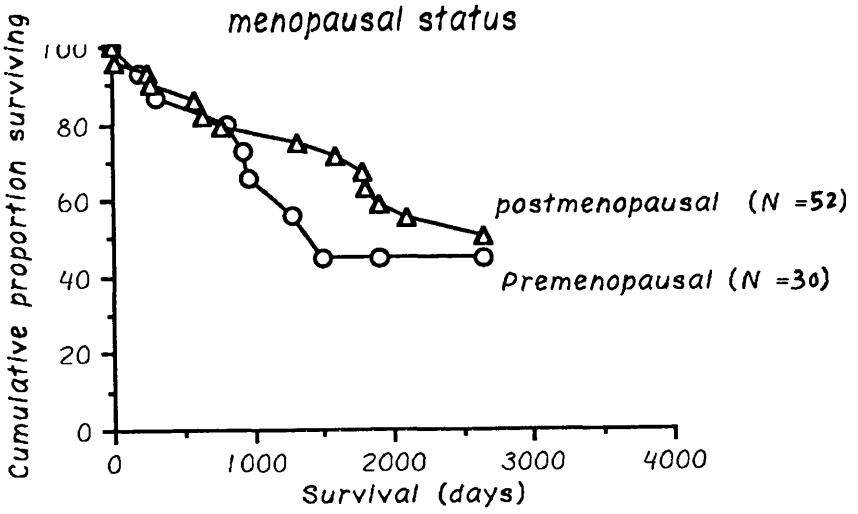


Table 34

Survival of patients with grade III tumours according to their menopausal status

Status	Survival	
	5-yr	10-yr
postmenopausal/GIII	59%	59%*
premenopausal/ GIII	45%	39%*

* by extrapolation

4.7.7. LN status correlated with survival

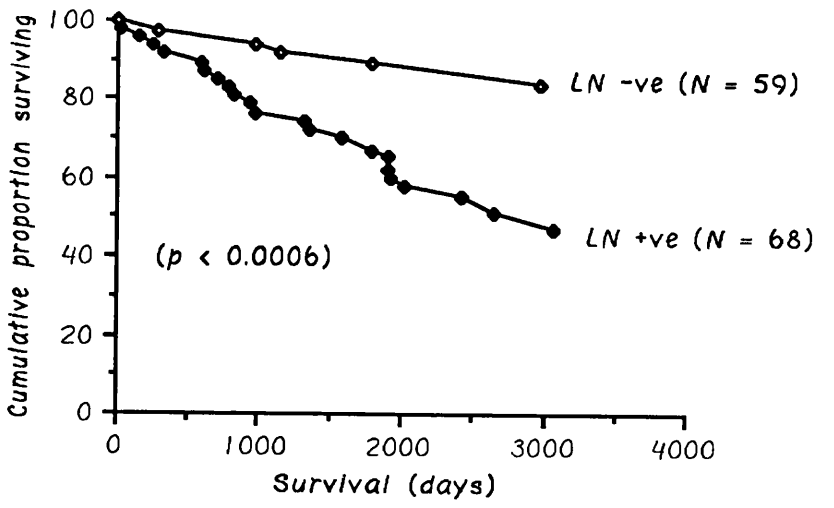
Information on LN status and survival was available on 127 cases. The results are presented in the following table (table; 35).

Table; 35

Survival of patients according to their LN status

Status	Total	Died BC	Died NBC	Alive	lost
LN+ve	68	22	0	16	30
LN-ve	59	4	1	24	30
Totals	127	26	1	40	60

fig. 107 Survival/ patients are divided according to their LN status (N = 127)



From the above life-table (Fig. 107), survival of patients according to their LN status, at 5- and 10-year intervals are presented in the following table (table; 36).

Table; 36

Survival rates of patients according to their LN status

Status	Survival		
	5-year	10-year	P-value
LN+ve	67%	47%*	< 0.0006
LN-ve	89%	84*	

* by extrapolation

Survival of patients at both 5- and 10-yr interval shows that those with LN involvement have much shorter survival (almost half) than those with no involvement.

4.7.8. Survival/ LN status according to presumed menopausal status

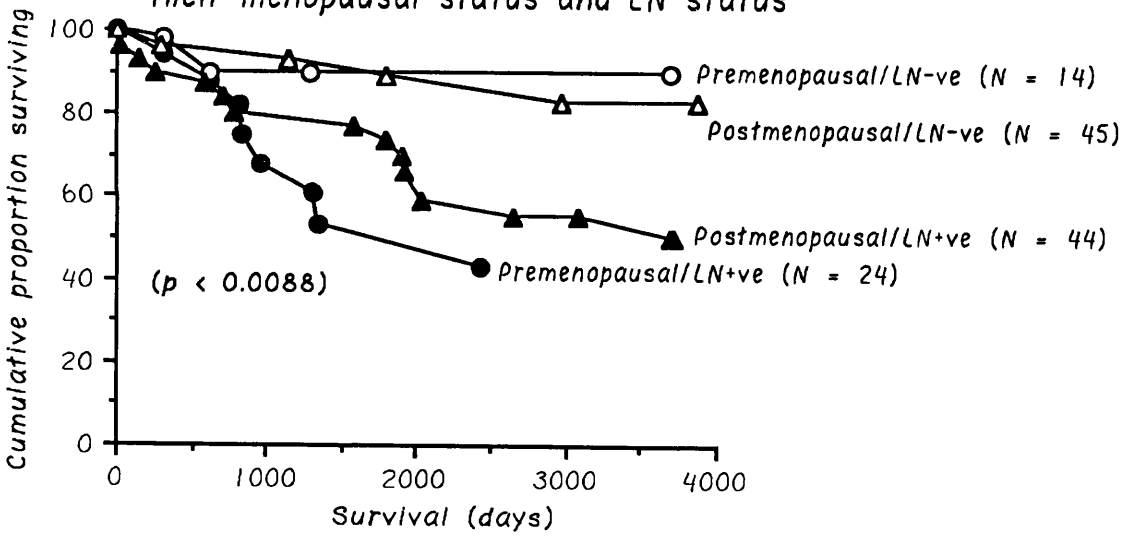
The results are presented in Table 37.

Table; 37

Survival of patients according to their menopausal and LN status

Status	Total	Died BC	Died NBC	Alive	Lost
postmenopausal/LN+	44	14	0	12	18
postmenopausal LN-	45	3	1	22	19
premenopausal LN+	24	8	0	4	12
premenopausal LN-	14	1	0	5	8
Totals	127	26	1	43	57

Fig. 108 Survival/ Patients are divided according to their menopausal status and LN status



From the above Fig. 108, survival rates of patients with different menopausal and LN status were obtained and these are presented in the following table.

Table; 38

Survival rates of patients according to their menopausal and LN status

Status	Survival 5-yr	10-yr	p-value
postmenopausal LN+	68%	50%	
postmenopausal LN-	88%	82%	
			< 0.0088
premenopausal LN+	48%	28%*	
premenopausal LN-	90%	90%	

* by extrapolation

4.7.9. Survival/ mistletoe lectins staining

4.7.9.1. Survival/ MLI staining

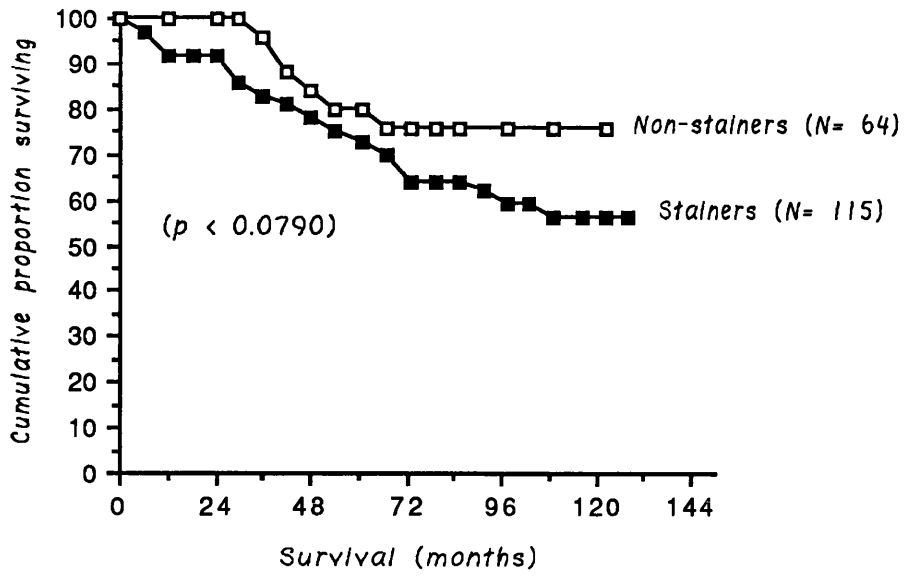
The staining for MLI was correlated with the survival of these patients (193). The staining results using MLI are shown in the following table:

Table; 39

Survival of patients according to their binding to MLI

MLI staining	Total	Died BC	Died NBC	Alive	Lost
+ve	115	25	1	25	64
-ve	64	6	0	16	42
Totals	179	31	1	41	106

Fig. 109 Survival/ MLI staining/ ~~All patients~~ (N=179)



The above Life-table (Fig. 109) shows that non-stainers for MLI survive longer than stainers ($p < 0.0790$). The rate of surviving of non-stainers and stainers at both 5- and 10 yrs is presented in the following table:

Table; 40

Survival rates of patients according to their binding to MLI

	Survival		
MLI staining	5 yrs	10 yrs	p-value
+ve	74%	55%	
			< 0.0790
-ve	80%	76%	

4.7.9.2. Survival/ MLI staining according to presumed menopausal status

This correlation was also done according to age (postmenopausal versus premenopausal, stainers versus non-stainers). The results for MLI in the 2 groups are presented in the following table (table; 41), where S= stainers and NS= non-stainers:

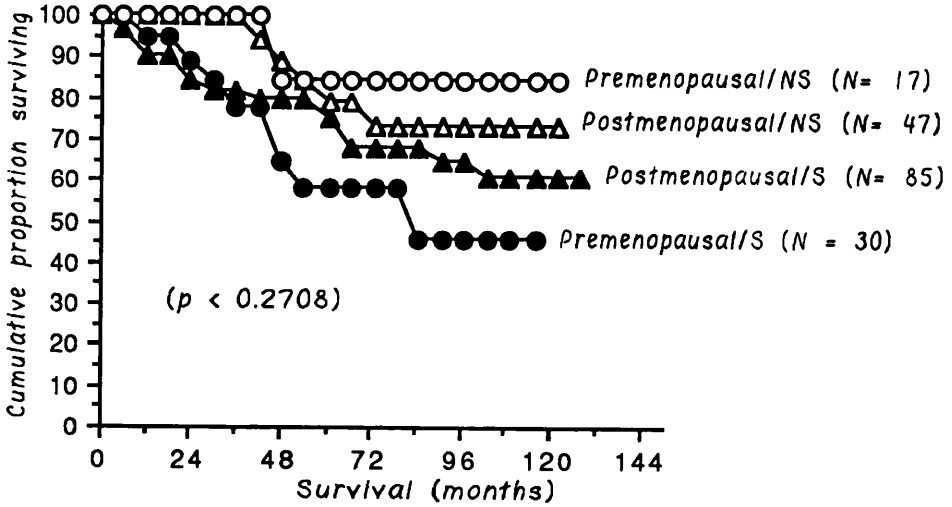
Table; 41

Survival of patients according to their binding to ML and their menopausal status

Status	Total	Died BC	Died NBC	Alive	Lost
postmenopausal NS	47	5	0	13	29
postmenopausal S	85	17	1	20	47
premenopausal NS	17	1	0	3	13
premenopausal S	30	8	0	5	17
Totals	179	31	1	41	106

Life-table (Fig. 110) shows survival rates in premenopausal and postmenopausal groups at 5 yrs and 10 yrs.

Fig. 110 Survival/ MLI staining/ Patients are divided into 2 age groups : Postmenopausal and premenopausal



Table; 42

Survival rates of patients according to their binding to ML and their menopausal status

Status	Survival 5-yr	10-yr	p-value
postmenopausal NS	81%	74%	
postmenopausal S	77%	61%	
			< 0.2708
premenopausal NS	84%	84%	
premenopausal S	60%	47%	

The survival analysis shows that while stainers in the premenopausal group do worse than stainers in the postmenopausal group, the non-stainers in premenopausal group do better than non-stainers in postmenopausal group at both 5- and 10-yr intervals.

4.7.9.3. Survival/ MLII staining

The results for MLII are shown in the following table Table; 43).

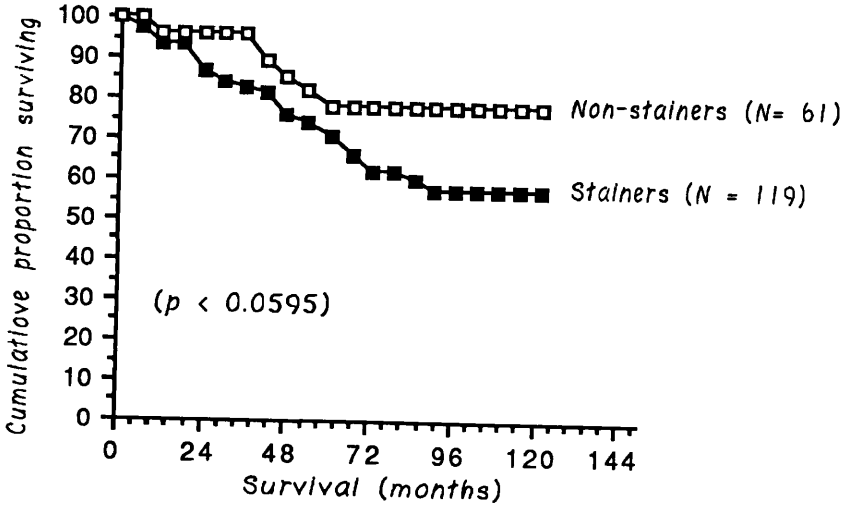
Table; 43

Survival of patients according to their binding to MLII

MLII staining	Total	Died BC	Died NBC	Alive	Lost
+ve	119	27	0	25	67
-ve	61	5	1	16	39
Totals	180	32	1	41	106

Fig. 111 shows that the non-stainers for MLII survive longer than the stainers. At 5 yrs , the rate of surviving of non-stainers is 80% persisted over 10 yrs, while the rate of surviving of stainers at 5 yrs is 72% and at 10 yrs is 55% ($p < 0.0595$).

Fig. 111 Survival/ MLII Staining/ All patients (180)



From Fig. 111, survival rates were obtained and these are presented in the following table.

Table; 44

Survival rates of patients according to their binding to MLII

MLII staining	Survival 5 yrs	10 yrs	p-value
+ve	72%	55%	
			< 0.0595
-ve	80%	80%	

4.7.9.4. Survival/ MLII staining according to presumed menopausal status

Results of survival analysis of cases stained for MLII divided into 2 age groups (postmenopausal and premenopausal) are presented in the following table (table; 45).

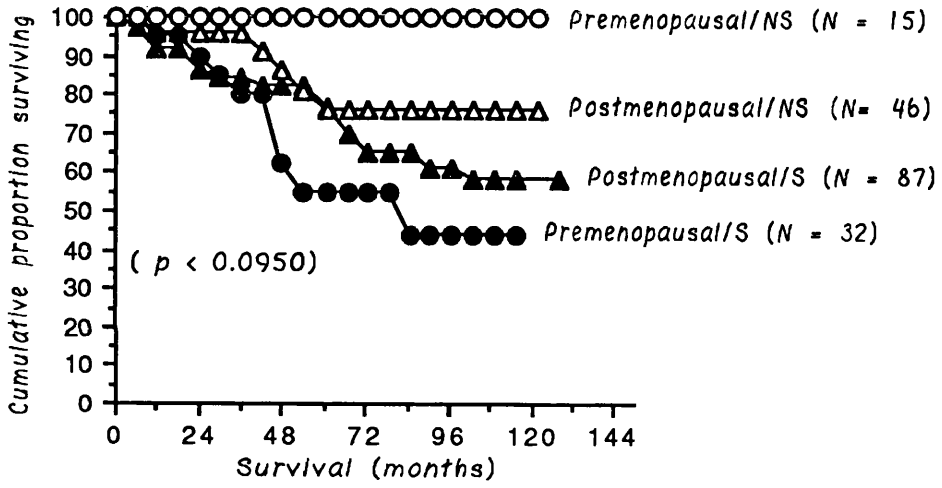
Table; 45

Survival of patients according to their binding to MLII and their menopausal status

Status	Total	Died BC	Died NBC	Alive	Lost
postmenopausal NS	46	5	1	13	27
postmenopausal S	87	18	0	20	49
premenopausal NS	15	0	0	3	12
premenopausal S	32	9	0	5	18
Totals	180	32	1	41	106

The following Fig. 112 shows survival rates in postmenopausal and premenopausal groups at 5 yrs 10 yrs:

Fig. 112 Survival / MLII staining/ Patients are divided into 2 age groups: Postmenopausal and premenopausal



Table; 46

Survival rates of patients according to their binding to MLII and their menopausal status

Status	Survival 5-yr	10-yr	p-value
postmenopausal NS	76%	76%	
postmenopausal S	76%	59%	
			< 0.0950
premenopausal NS	100%	100%	
premenopausal S	56%	43%	

As with MLI, stainers in premenopausal group do worse than those in postmenopausal group. Also non-stainers if they were premenopausal, do better than postmenopausal.

4.7.9.5. Survival/ MLIII staining

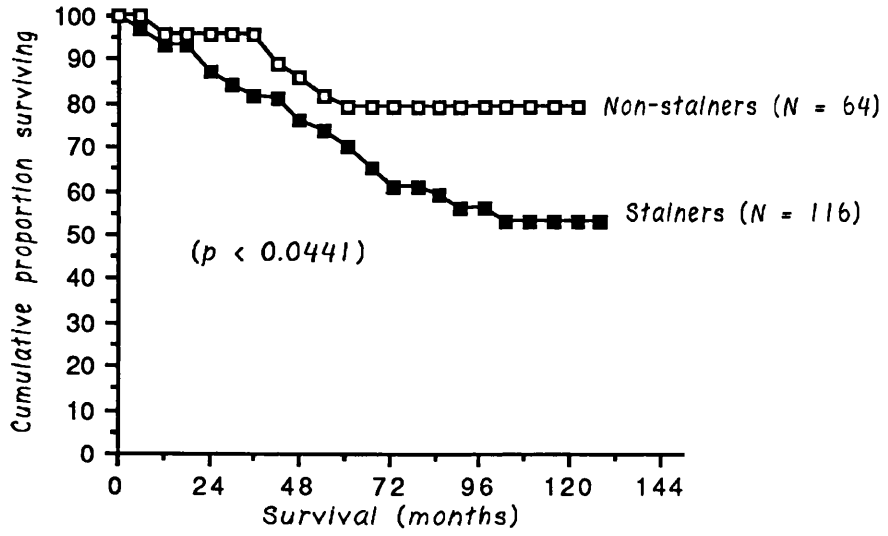
The results for MLIII are shown in the following table (table; 47).

Table; 47
Survival of patients according to their binding to MLIII

MLIII staining	Total	Died BC	Died NBC	Alive	Lost
+ve	116	27	0	25	64
-ve	64	5	1	16	42
Totals	180	32	1	41	106

The following Life-table (Fig. 113) shows that the non-stainers for MLIII have a higher survival rate than the stainers. This difference between 2 groups only just reached statistical significance ($p < 0.0441$).

Fig. 113 Survival/ MLIII staining/ All patients (N=180)



Survival rates presented in the following table were obtained from Fig. 113

Table; 48

Survival rates of patients according to their binding to MLIII

MLIII staining	Survival 5 yrs	10 yrs	p-value
+ve	71%	54%	
			< 0.0441
-ve	80%	76%	

4.7.9.6. Survival/ MLIII staining according to menopausal status

The following table (table; 49) outlines the results of survival analysis in postmenopausal and premenopausal groups stained for MLIII:

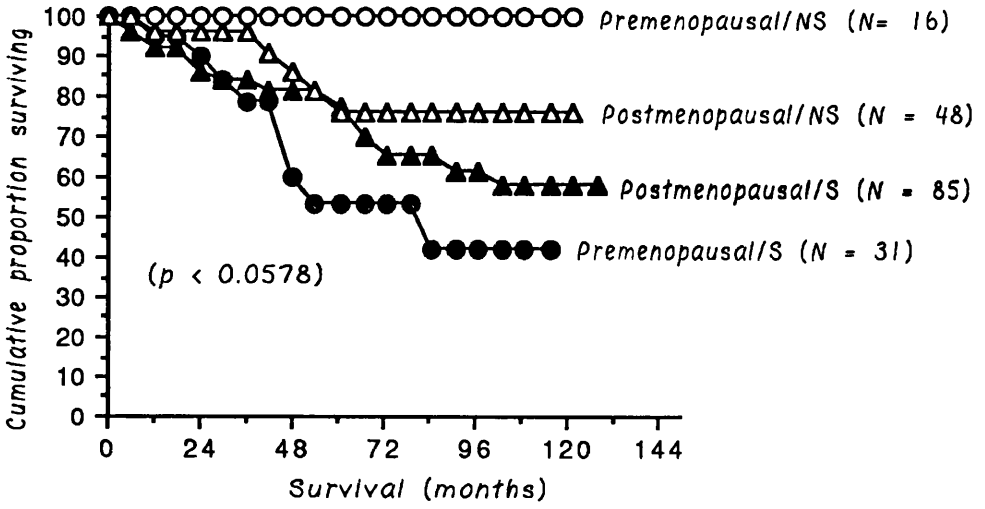
Table; 49

Survival of patients according to their binding to MLIII and their menopausal status

Status	Total	Died BC	Died NBC	Alive	Lost
postmenopausal NS	48	5	1	13	29
postmenopausal S	85	18	0	20	47
premenopausal NS	16	0	0	3	13
premenopausal S	31	9	0	5	17
Totals	180	32	1	41	106

Fig. 114 shows the rate of survival in postmenopausal and premenopausal groups

Fig. 114 Survival/ MLIII staining/ Patients are divided into 2 age groups: postmenopausal and premenopausal



Survival rates are presented in table; 50.

Table; 50

Survival rates of patients according to their binding to MLIII and their menopausal status

Status	Survival 5-yr	10-yr	p-value
postmenopausal NS	75%	75%	
postmenopausal S	77%	58%	
			< 0.0578
premenopausal NS	100%	100%	
premenopausal S	53%	42%	

From the survival rates in the tables presented above, it appears that premenopausal patients do worse if they were stainers for MLIII than if they were stainers for MLII or MLI. The difference in survival rate between stainers and non-stainers for MLIII is larger than what has been seen with MLII and with MLI.

Tabulated summary of results of survival analysis are presented in the following table.

Table; 51

	Survival 5-year	10-year	P-value
<2cm	88%	72%*	
2-5cm	82%	52%*	< 0.00001
>5cm	20%	0	
G II	100%	100*	
			< 0.0191
G III	57%	37%	
LN+ve	67%	47%*	
			< 0.0006
LN-ve	89%	84%*	
MLI/S	74%	55%	
			< 0.0790
MLI/NS	80%	76%	
MLII/S	72%	55%	
			< 0.0595
MLII/NS	80%	80%	
MLIII/S	71%	54%	
			< 0.0441
MLIII/NS	80%	80%	

* by extrapolation S = Stainers
NS = Non-stainers G = Grade
LN = lymph node

Survival analysis according to menopausal status are presented in the following table.

Table; 52

Survival/ tumour size, grade, LN status and staining of MLI, MLII & MLIII according to menopausal status Status

	Survival 5-yr	10-yr
postmenopausal/<2cm	88%	82%
postmenopausal/2-5cm	83%	68%
premenopausal/<2cm	78%	76%
premenopausal/2-5cm	60%	51%
postmenopausal/GIII	59%	59%*
premenopausal/ GIII	45%	39%*
postmenopausal LN-	88%	82%
postmenopausal LN+	68%	50%
premenopausal LN-	90%	90%
premenopausal LN+	48%	28%*
MLI		
postmenopausal NS	76%	76%
postmenopausal S	76%	59%
premenopausal NS	100%	100%
premenopausal S	56%	43%
MLII		
postmenopausal NS	81%	74%
postmenopausal S	77%	61%
premenopausal NS	84%	84%
premenopausal S	60%	47%
MLIII		
postmenopausal NS	75%	75%
postmenopausal S	77%	58%
premenopausal NS	100%	100%
premenopausal S	53%	42%

* by extrapolation NS = Non-stainers
S = Stainers G = Grade LN = lymph node

4.8. correlation of staining with Disease Free Interval

4.8.1. Disease free interval (DFI)

The time between surgery (ie, histological confirmation of cancer) and confirmation of disease spread (for example, by bone scan, biopsy), was taken as time to first recurrence. Time to first recurrence is referred to as disease free interval (DFI). Times were recorded in months. Recurrences included local (chest wall and glandular), regional (including pleural effusion, skin elsewhere, and other breast), and distant (bone, liver, lung, and brain). This information was obtained from patients notes.

When the patients were lost to follow up immediately after surgery, their DFIs were calculated as one day after the date of operation or biopsy. When the patients were lost to follow-up sometime after surgery, their DFIs were calculated as one day after the date they were last seen. Including these patients was essential to reduce bias of statistical analysis.

The DFI of 193 patients was analysed and the results show that at follow-up of minimum period of 9 yrs and maximum of 11 yrs, 104 patients were lost to follow-up, 52 recurred and 37 stayed with no sign of recurrence (NSR).

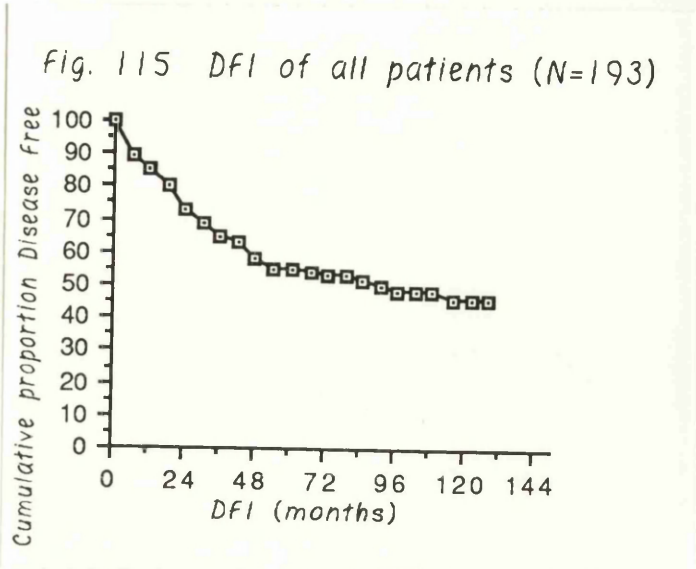


Fig. 115 show that at 5 yrs, the cumulative proportion disease free is 55% and at 10 yrs is 46% ((table; 53).

Table; 53

DFI of all patients (N=193)

5 yrs	10 yrs
55%	46%

4.8.2. DFI of patients according to menopausal status

When the 193 cases were divided into two age groups equal and less than 50 yrs old (premenopausal) and over 50 yrs old (postmenopausal), the number of patients in the younger group was 51 of whom 20 recurred , 6 still with NSR, and 25 were lost to follow-up. The number of patients in the older group was 142 of whom 32 recurred, 31 still with NSR and 79 were lost to follow-up (Table, 54)

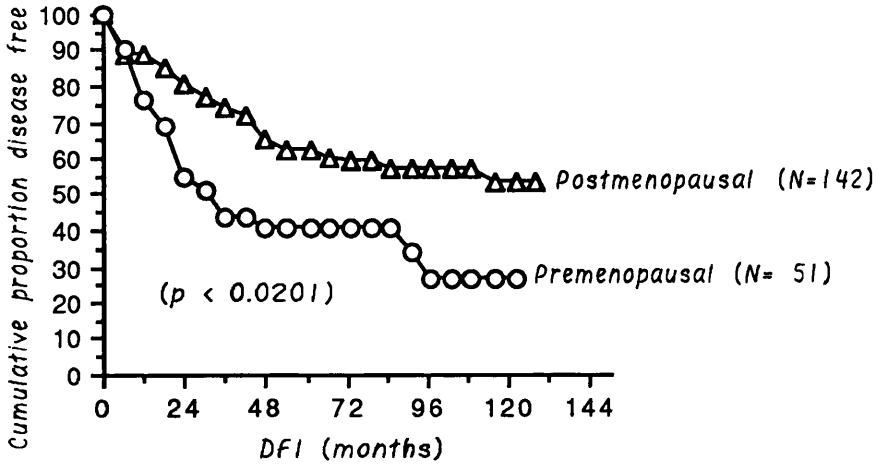
Table; 54

DFI of patients according to their menopausal status

Status	Total	Recurred	NSR	Lost
Premenopausal	51	20	6	25
Postmenopausal	142	32	31	79
Totals	193	52	37	104

There was a significant difference between the 2 groups in DFI ($p < 0.0201$) (Fig. 116)

Fig. 116 DFI of the 193 patients divided into 2 age groups: postmenopausal and premenopausal



The above presented data suggest that the probability of recurrence in the older patients is lower than those younger. Premenopausal patients might recur sooner as outlined in the following table.

Table; 55

Cumulative proportion disease free of patients according to their menopausal status

status	DFI		
	5 yrs	10 yrs	p-value
Premenopausal	41%	27%	
			< 0.0201
Postmenopausal	65%	53%	

4.8.3. Primary tumour size correlated with DFI

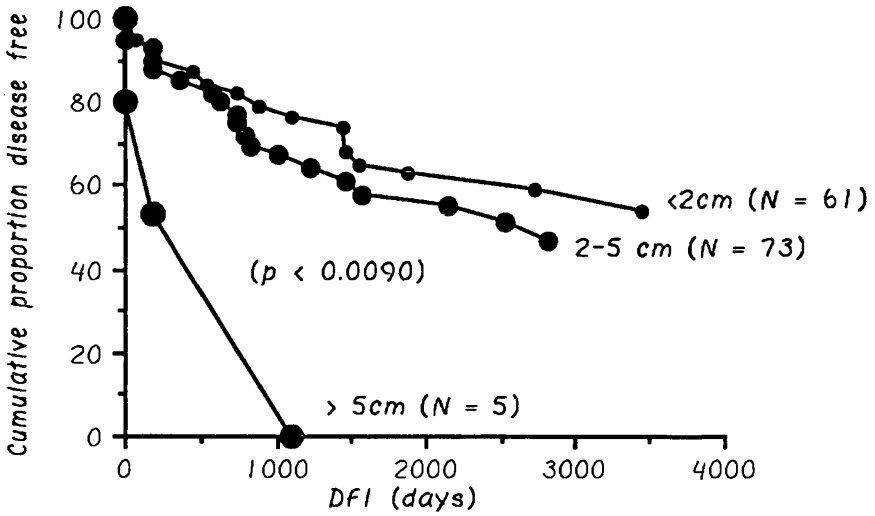
Information on primary tumour size and DFI were available on 139 cases. The results are presented in the following table:

Table; 56

DFI of patients according to their 1^{o} tumour size

1^{o} size	Total	Recurred	NSR	lost
<2cm	61	16	19	26
2-5cm	73	20	15	38
>5cm	5	3	0	2
Totals	139	39	34	66

Fig. 117 DFI patients are divided according to their primary tumour size (N = 139)



From the above Fig. 117 , DFI of patients according to their tumour size, at 5- and 10-year intervals are presented in the following table:

Table; 57

Cumulative proportion disease free of patients according to their 1° tumour size

	DFI		
1°size	5-year	10-year	P-value
<2cm	62%	51%	
2-5cm	58%	37%*	
			< 0.0090
>5cm	0	0	

* by extrapolation

Only 5 patients had tumour size larger than 5cm. These patients recurred earlier than 5-year interval. DFI analysis suggests that the larger the tumour size, the higher the recurrence rate.

4.8.4. Primary tumour size/DFI correlated with menopausal status

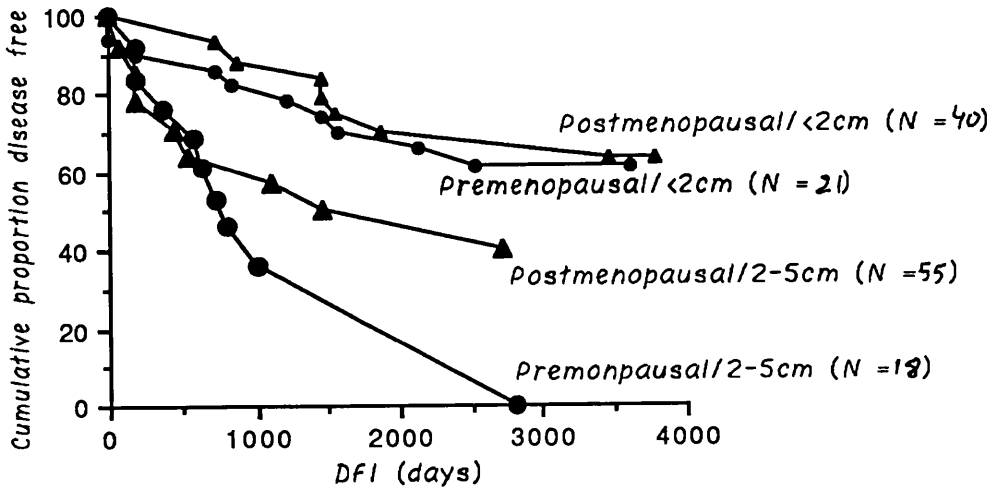
Table; 58

DFI of patients according to their menopausal status and their 1^{o} tumour size

Status	Total	Recurred	NSR	Lost
postmenopausal/<2	40	8	15	27
postmenopausal/2-5	55	11	13	31
Postmenopausal/>5	5	3	0	2
premenopausal/<2	21	8	4	9
premenopausal/2-5	18	9	2	7
Totals	149	39	34	76

the following life-table (Fig. 118) shows that the larger the tumour size, the shorter the DFI, especially in premenopausal patients.

Fig. 118 DFI/ patients are divided according to their tumour size and menopausal status



Cumulative proportion disease free interval of patients are presented in the following table:

Table; 59

Cumulative proportion disease free of patients according to their menopausal status and their 1° tumour size

	5-yr	10-yr
postmenopausal <2cm	72%	62%
postmenopausal 2-5cm	47%	28%*
premenopausal <2cm	68%	60%
premenopausal 2-5cm	18%	0%

4.8.5. Tumour grade correlated with DFI

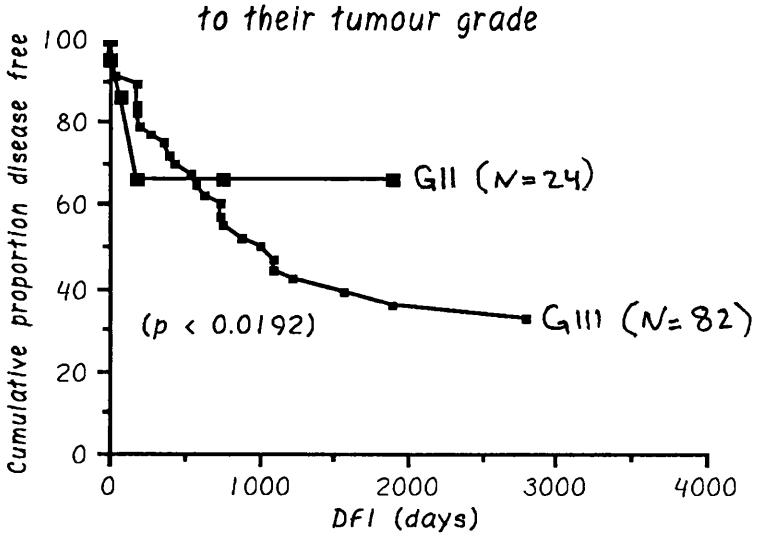
Information on tumour grade and DFI were available on 113 cases. The results are presented in the following table (table; 60), where G = Grade:

Table; 60

DFI of patients according to their tumour grade

Grade	Total	Recurred	NSR	lost
G III	82	28	11	43
G II	24	2	6	16
G I	7	1	0	6
Totals	113	31	17	65

Fig. 119 DFI/ Patients are divided according to their tumour grade



From Fig. 119, it appears that recurrence rate of patients with high tumour grade is higher than those with lower tumour grade at both 5- and 10-year intervals (table; 61).

Table; 61

Cumulative proportion disease free of patients according to their tumour grade

	DFI		
Grade	5-year	10-year	P-value
G II	66%	66%*	< 0.0192
G III	35%	29%	

* by extrapolation

4.8.6. Tumour grade/DFI correlated with menopausal status

The results are tabulated below (table; 62).

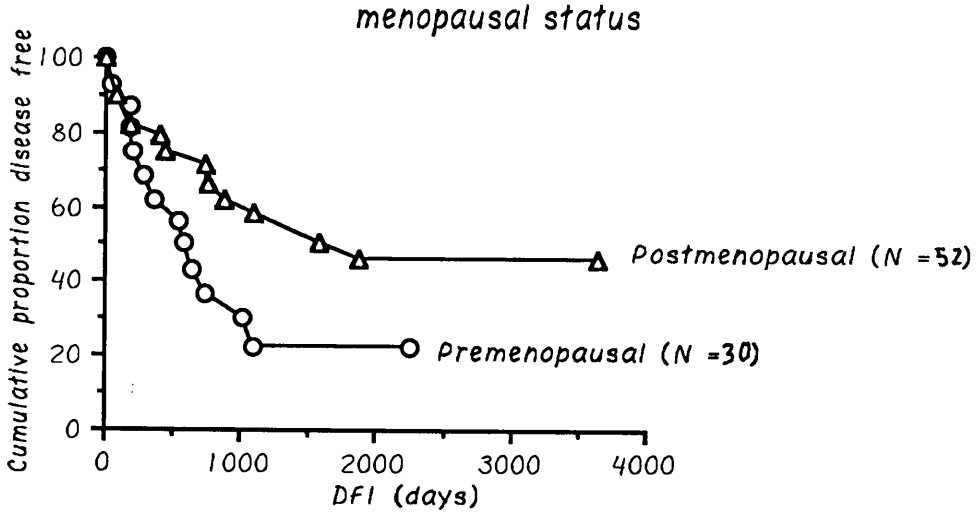
Table; 62

DFI of patients according to their menopausal status and tumour grade

Status	Total	Recurred	NSR	Lost
postmenopausal/GI	4	0	0	4
postmenopausal/GII	20	1	5	14
Postmenopausal/GIII	52	16	10	26
premenopausal/GI	3	1	0	2
premenopausal/GII	4	1	1	2
Premenopausal/GIII	30	12	1	17
Totals	113	31	17	65

In the following life-table (Fig. 120) , only those with GIII tumours were shown. This is owing to the low number of patients in the other two grades.

Fig. 120 DFI patients with poorly differentiated tumours divided into 2 groups according to their menopausal status



Table; 63

Cumulative proportion disease free of patients according to their menopausal status and tumour grade

Status	DFI 5-yr	10-yr
postmenopausal GIII	48%	48%
premenopausal GIII	22%	22%*

* by extrapolation

4.8.7. LN status correlated with DFI

information on LN status and DFI were available on 127 cases. The results of the correlation are presented in the following table:

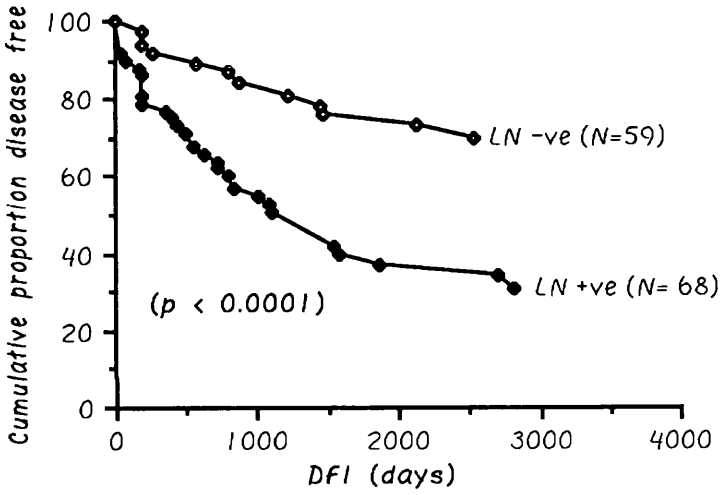
Table; 64

DFI of patients according to their LN status

Status	Total	Recurred	NSR	lost
LN+ve	68	33	12	23
LN-ve	59	11	24	24
Totals	127	44	36	47

From the following life-table (Fig. 121), DFI of patients according to their LN status, at 5- and 10-year intervals are presented in table; 65.

Fig. 121 DFI/ patients are divided according to their LN status (No. = 127)



Table; 65

Cumulative proportion disease free of patients according to their LN status

	DFI		
Status	5-year	10-year	P-value
LN+ve	36%	27%*	< 0.0001
LN-ve	75%	72%*	

* by extrapolation

The DFI of patients with LN+ve is much shorter than those with LN-ve at both 5- and 10-year intervals.

4.8.8. DFI/ LN staus according to menopausal status

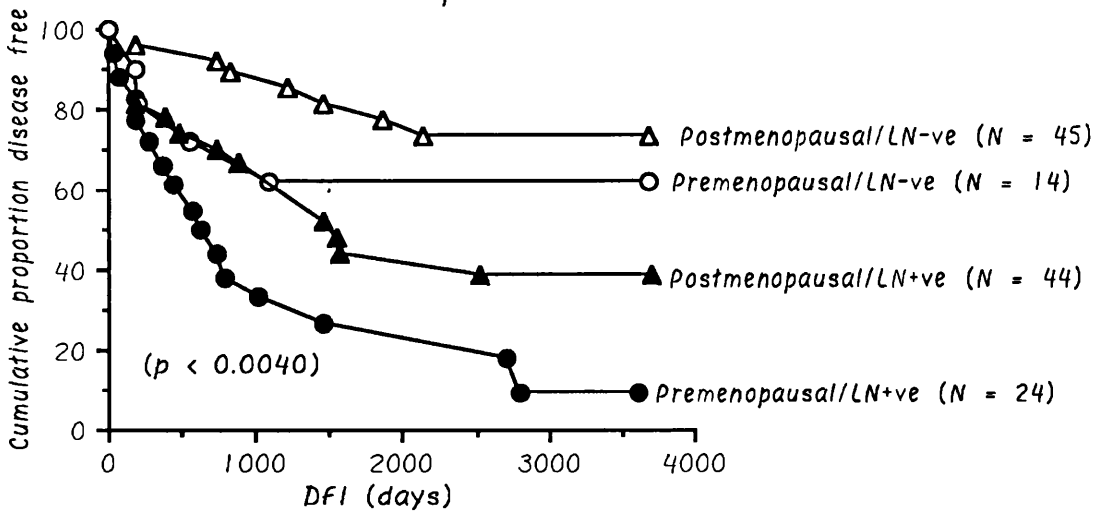
The results are presented in the following table.

Table; 66

DFI of patients according to their menopausal and LN status

Status	Total	Recurred	NSR	Lost
postmenopausal LN+	44	18	10	16
postmenopausal LN-	45	7	20	18
premenopausal LN+	24	15	2	7
premenopausal LN-	14	4	4	6
Totals	127	44	36	47

Fig. 122 DFI/ patients are divided according to their menopausal status and LN status



From Fig. 122, the following cumulative proportion disease free intervals were obtained (table; 67).

Table; 67

Cumulative proportion disease free of patients according to their menopausal and LN status

	DFI		
Status	5-yr	10-yr	p-value
postmenopausal LN+	43%	38%	
postmenopausal LN-	78%	75%	
			< 0.0040
premenopausal LN+	25%	9%	
premenopausal LN-	62%	62%	

4.8.9. DFI/ mistletoe lectin staining

4.8.9.1. DFI/MLI staining

The staining for MLI was correlated with DFI. The results for MLI are shown in the following table.

Table; 68

DFI of patients according to their binding to MLI

MLI staining	Total	Recurred	NSR	lost
+ve	115	39	21	55
-ve	64	7	16	41
Totals	179	46	37	96

Fig. 123 DFI/ MLI staining/All patients (N=179)

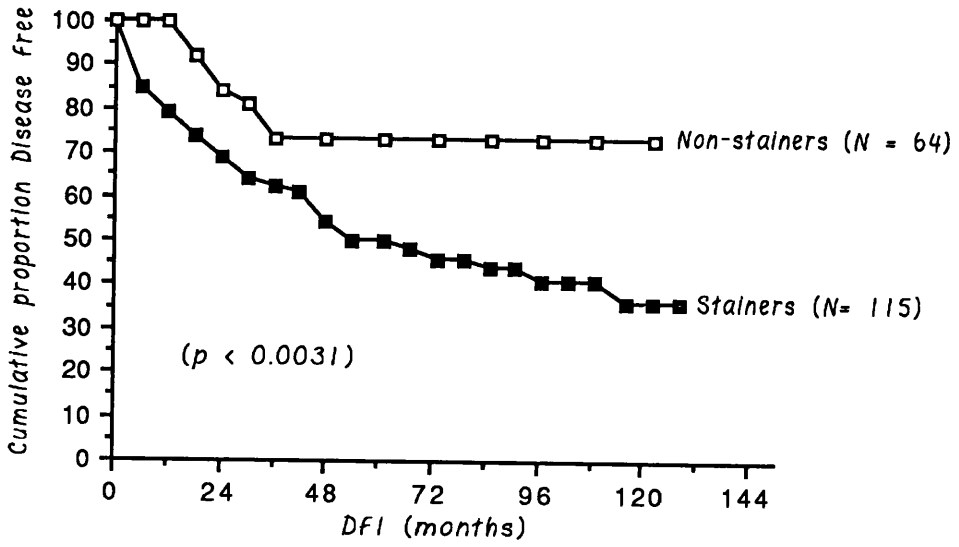


Fig. 123 shows that 73% of non-stainers are disease free at 5 yrs and the rate persisted over a period of 10 yrs. 52% of stainers for MLI are still disease free at 5 yrs while at 10 yrs this percent went down to only 36%.

There was a significant difference between the groups with and without MLI staining ($P < 0.0031$) (table; 69).

Table; 69

Cumulative proportion disease free of patients according to their binding to MLI

	DFI		
MLI staining	*5 yrs*	*10 yrs*	*p-value*
+ve	52%	36%	
			< 0.0031
-ve	73%	73%	

4.8.9.2. DFI/ MLI staining according to presumed menopausal status

Results are presented in the following table,

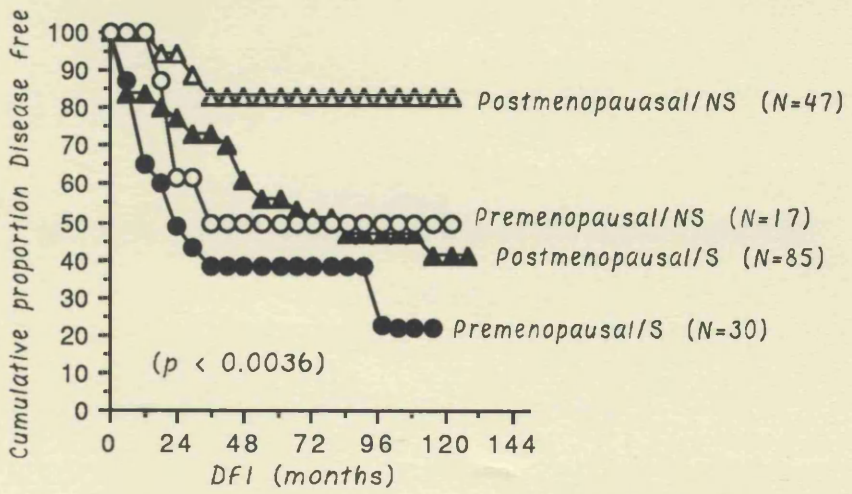
Table; 70

DFI of patients according to their binding to MLI and their menopausal status

Status	Total	Recurred	NSR	Lost
postmenopausal NS	47	3	13	31
postmenopausal S	85	26	18	41
premenopausal NS	17	4	3	10
premenopausal S	30	13	3	14
Totals	179	46	37	96

A significant difference was found between the 2 groups with and without MLI staining ($p < 0.0036$).

Fig . 124 shows that in the postmenopausal group, at 5 yrs, 83% of non-stainers and 56% of stainers are still disease free. At 10 yrs the rate of non-stainers being disease stayed the same while of stainers went down to 41%. In the premenopausal group, the rate of being disease free, of non-stainers is 50% at both 5 and 10 yrs. For stainers the rate decreased from 38% at 5 yrs to 22% at 10 yrs.



Table; 71

Cumulative proportion disease free of patients according to their binding to MLI and their menopausal status

Status	DFI 5-yr	10-yr	p-value
postmenopausal NS	83%	83%	
postmenopausal S	56%	41%	
			< 0.0036
premenopausal NS	50%	50%	
premenopausal S	38%	22%	

Stainers for MLI do worse than non-stainers whether they are pre- or post-menopausal.

Stainers in premenopausal group do worse than stainers in postmenopausal group.

Premenopausal patients whether they are stainers or non-stainers do worse than postmenopausal patients.

4.8.9.3. DFI/ MLII staining

The results for MLII are shown in the following table.

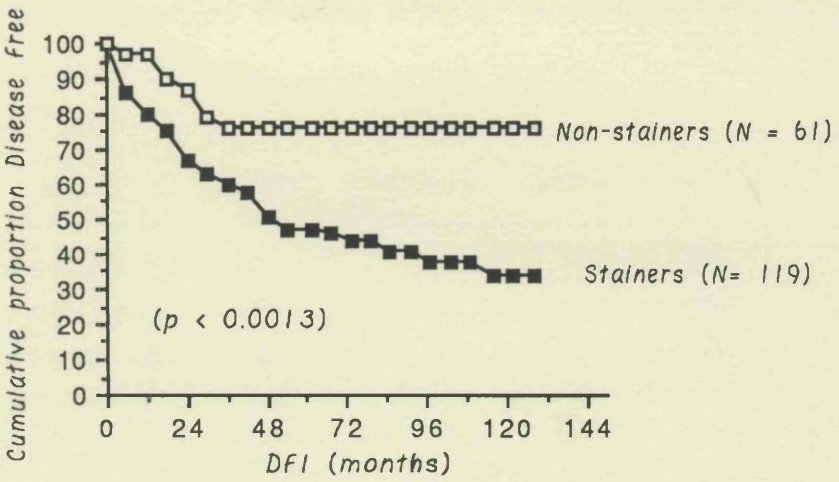
Table; 72

DFI of patients according to their binding to MLII

MLII staining	Total	Recurred	NSR	lost
+ve	119	40	19	60
-ve	61	7	18	36
Totals	180	47	37	96

Fig. 125 (also table; 73) show that at 5 yrs 76% of non-stainers for MLII are still disease free while of stainers 47% are still disease free, and at 10 yrs, the same rate for non-stainers persisted while for stainers decreased to 35%. The differences presented above were statistically significant ($p < 0.0013$).

Fig. 125 DFI/ MLII staining/ All patients (N=180)



Table; 73

Cumulative proportion disease free of patients according to their binding to MLII

	DFI		
MLII staining	5 yrs	10 yrs	p-value
+ve	47%	35%	
			< 0.0013
-ve	76%	76%	

4.8.9.4. DFI/ MLII staining according to menopausal status

Results are presented in the following table.

Table; 74

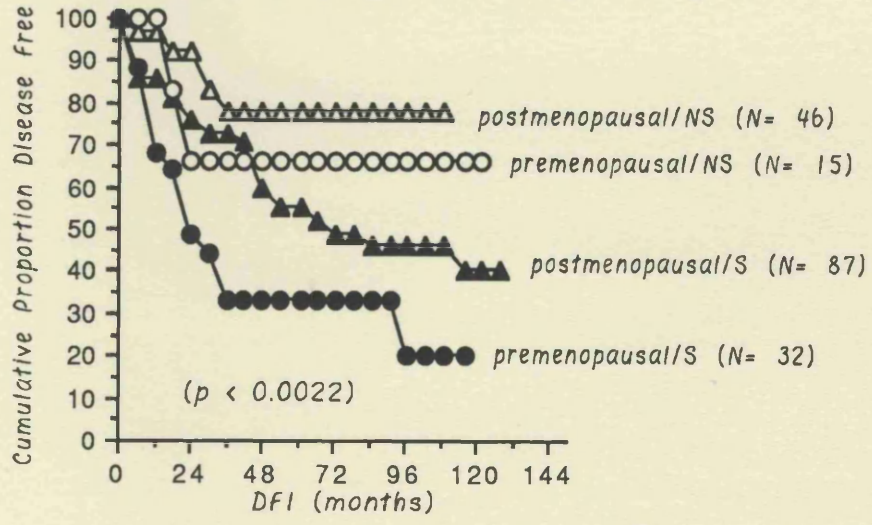
Cumulative proportion disease free of patients according to their binding to MLII and their menopausal status

Status	Total	Recurred	NSR	Lost
postmenopausal NS	46	5	15	26
postmenopausal S	87	25	16	46
premenopausal NS	15	2	3	10
premenopausal S	32	15	3	14
Totals	180	47	37	96

A significant difference was found between the groups with and without MLII staining ($P < 0.0022$).

Fig. 126 shows that 78% of postmenopausal patients that did not stain for MLII are disease free at both 5 and 10 yrs. For stainers a rate of 56% at 5 yrs that went down to 44% at 10 yrs was found.

Fig. 126 DFI/ MLII staining/ Patients are divided into 2 age groups: postmenopausal and premenopausal



In the premenopausal group, the rate of patients being disease free of non-stainers was the same at 5 and at 10 yrs (66%). For stainers the 33% of patients that were disease free at 5 yrs decreased to only 21% at 10 yrs (table; 75).

Table; 75

Cumulative proportion disease free of patients according to their binding to MLII and their menopausal status

	DFI		
Status	*5-yr*	*10-yr*	*p-value*
postmenopausal NS	*78%*	*78%*	
postmenopausal S	*56%*	*44%*	
			< 0.0022
premenopausal NS	*66%*	*66%*	
premenopausal S	*33%*	*21%*	

The difference in DFI between the 2 age groups is greater than that seen with MLI.

4.8.9.5. DFI/ MLIII staining

The results for MLIII are shown in the following table.

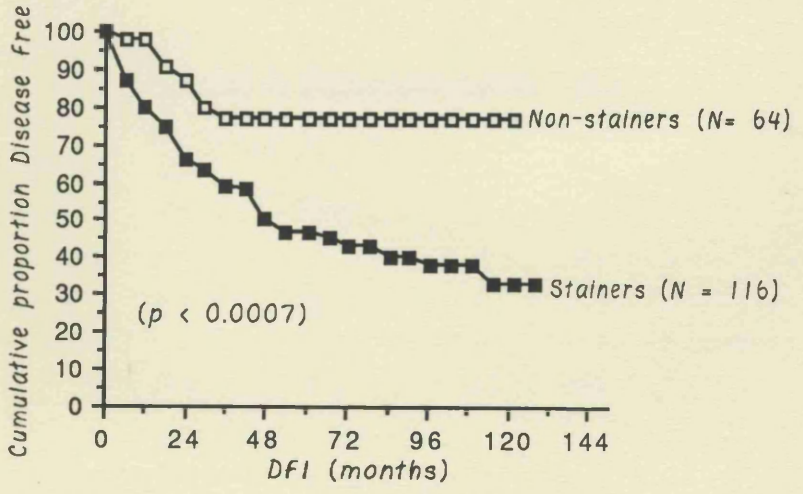
Table; 76

DFI of patients according to their binding to MLIII

MLIII staining	Total	Recurred	NSR	lost
+ve	116	40	19	57
-ve	64	7	18	39
Totals	180	47	37	96

Fig. 127 show that at 5 yrs, 77% of non-stainers are disease free. This rate persisted over a period of 10 yrs. For MLIII-stainers, the 39% that were disease free at 5 yrs went even lower to 33% at 10 yrs. The difference between the 2 groups showed a p-value of < 0.0007 (the results are tabulated in table; 77).

Fig. 127 DFI/ MLIII staining/ All patients (N = 180)



Table; 77

Cumulative proportion disease free of patients according to their binding to MLIII

	DFI		
MLIII staining	5 yrs	10 yrs	p-value
+ve	39%	33%	< 0.0007
-ve	77%	77%	

4.8.9.6. DFI/ MLIII staining according to presumed menopausal status

The results are presented in the following table.

Table; 78

DFI of patients according to their binding to MLIII and their menopausal status

Status	Total	Recurred	NSR	Lost
postmenopausal NS	48	5	15	28
postmenopausal S	85	25	16	44
premenopausal NS	16	2	3	11
premenopausal S	31	15	3	13
Totals	180	47	37	96

A significant difference was found between the different groups with and without MLIII staining ($p < 0.0007$).

Fig. 128 DFI/ MLIII staining/ Patients are divided into 2 age groups: postmenopausal and premenopausal

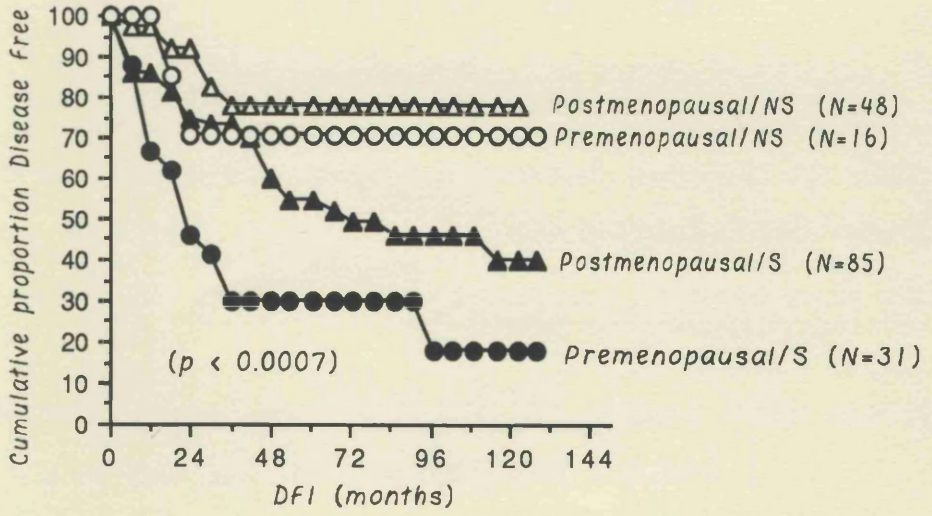


Fig. 128 shows that 78% of non-stainers in the postmenopausal group, stayed disease free over a period of 10 yrs. For stainers the rate of being disease free went from 56% at 5 yrs to 40% at 10 yrs.

In the premenopausal group, 71% of non-stainers were disease free at 5 and 10 yrs. The stainers rate of being disease free went from 31% at 5 yrs to 18% at 10 yrs Table; 79).

binding to MLIII and menopausal status

Status	DFI 5-yr	10-yr	p-value
postmenopausal NS	78%	78%	
postmenopausal S	56%	40%	
			< 0.0007
premenopausal NS	71%	71%	
premenopausal S	31%	18%	

Only 18% of premenopausal patients that stained positively for MLIII are disease free at 10 years, compared to 71% of non-stainers. This rate (18%) is less than half the rate seen in stainers in postmenopausal group (40%).

The difference between the groups with and without MLIII staining is more significant than that seen with MLII and with MLI.

Tabulated summary of results of correlation

Table; 80

Status	DFI 5-year	10-year	P-value
<2cm	62%	51%	
2-5cm	58%	37%*	< 0.0090
>5cm	0	0	
G II	66%	66%*	
			< 0.0192
G III	35%	29%*	
LN+ve	36%	27%*	
			< 0.0001
LN-ve	75%	72%*	
MLI/S	52%	36%	
			< 0.0031
MLI/NS	73%	73%	
MLII/S	47%	35%	
			< 0.0013
MLII/NS	76%	76%	
MLIII/S	39%	33%	
			< 0.0007
MLIII/NS	77%	77%	

* by extrapolation S = Stainers
NS = Non-stainers G = Grade
LN = lymph node

Table; 81

DFI/ LN status/ staining with MLI, MLII & MLIII according to menopausal status Status

Status	DFI 5-yr	10-yr
postmenopausal <2cm	72%	62%
postmenopausal 2-5cm	47%	28%*
premenopausal <2cm	68%	60%
premenopausal 2-5cm	18%	0%
postmenopausal GIII	48%	48%
premenopausal GIII	22%	22%*
postmenopausal LN-	78%	75%
postmenopausal LN+	43%	38%
premenopausal LN-	62%	62%
premenopausal LN+	25%	9%
MLI		
postmenopausal NS	83%	83%
postmenopausal S	56%	41%
premenopausal NS	50%	50%
premenopausal S	38%	22%
MLII		
postmenopausal NS	78%	78%
postmenopausal S	56%	44%
premenopausal NS	66%	66%
premenopausal S	33%	21%
MLIII		
postmenopausal NS	78%	78%
postmenopausal S	56%	40%
premenopausal NS	71%	71%
premenopausal S	31%	18%

* by extrapolation NS = Non-stainers
S = Stainers G = Grade LN = lymph node

DISCUSSION

Alterations in the oligosaccharide component of glycolipids and glycoproteins of malignant cells when compared to those of normal cells are well documented (Hakomori, 1989). Such modifications may contribute to the changes in cell growth and cell control which are a feature of neoplasia, alterations in these properties could be highly significant in the development and progression of the neoplastic process (Fidler, 1976; Nicolson, 1988a, 1988b).

Lectins, because of their affinities for specific sugars, have proved to be useful tools for the investigation of cell membranes of normal and malignant cells (Leathem and Gardner, 1981; Lis and Sharon, 1986; Leathem, 1986).

In this study attempts were made to isolate the lectins from mistletoe, to examine the place of these lectins in cancer treatment and their use as a tissue-based predictive test for biological behaviour of breast cancer.

Isolation of mistletoe lectins

The isolation carried out in London using mistletoe grown on apple tree was found in this study to contain only 1 lectin. The lectin was isolated by making a crude extract of the mistletoe plant, precipitation of the proteins, gel filtration, ion-exchange and affinity chromatography.

The intact lectin migrated in PAGE (without reducing agent) in the presence of SDS corresponding to a MW of 60KD. In addition 2 fainter bands were also found corresponding to MWs of 29KD and 34KD. The reduced lectin (when Mercaptoethanol was included in the sample buffer) gave rise to 2 major bands corresponding to MW of 29KD and 34KD.

By haemagglutination inhibition the lectin was found to have a major affinity for the simple sugar Galactose.
This is in agreement with Olsnes et al (1982) who isolated only one lectin and called the lectin Viscumin. However the work done in East Berlin confirmed the presence of 3 mistletoe lectins, although yields of isolated lectin were different. The mistletoe used there was grown on the Locust tree (Robinia pseudoacacia) and differences in the number and yield of lectins isolated could be influenced by differences in host tree, the type or sub-species of the mistletoe and the season of harvesting (Franz, 1985). It is possible that differences reported here may be caused by one or more of these factors.

In this study the isolation procedure used was different from that of Franz, et al (1981), who isolated 3 lectins (MLI, MLII & MLIII) and of Samtleben and Kiefer (1985), who isolated 2 lectins (VAAI & VAAII). The difference in isolation procedure used might also account for the differences in results obtained. However, the main lectin in mistletoe is the galactose specific lectin (MLI; VAAI); the total quantity of MLII and MLIII in mistletoe extract amounts to only about 15% of the total quantity of MLI (Ziska and Franz, 1985) so it is possible that they were lost in the isolation procedure as the sensitivity level of detection was low (haemagglutination test).
There is no doubt that the lectin isolated in this study is identical to MLI (Franz et al, 1981), VAAI (Samtleben and Kiefer, 1985) and Viscumin (Olsnes, et al, 1982) but the relatioship of MLII and MLIII to VAAII is not clear.

Characteristics of the 3 Lectins isolated.

MLII and MLIII are very close to each other in respect of charge and

molecular weight (the intact MLII and the intact MLIII by PAGE migrated corresponding to a MW of 56KD-58KD, the reduced MLII and the reduced MLIII each gave rise to 2 main bands of MW of 30-32KD and 25-27KD).

The major sugar specificity of the 3 mistletoe lectins, MLI, MLII & MLIII, to Galactose, N-Acetyl-Galactosamine (GalNAc) and GalNAc respectively is in agreement with Franz, et al (1981). This carbohydrate specificity was determined by haemagglutination inhibition.
On tissues, the 3 mistletoe lectins used in this study were inhibited by both galactose and GalNAc; MLI inhibited strongly by galactose and partially by GalNAc; MLII and MLIII inhibited strongly by GalNAc and partially by galactose.
None of the lectins was inhibited completely on tissues by their corresponding monosaccharide sugars. This may be because perhaps lectins prefer to bind to more complex carbohydrate in tissues. However complete inhibition was achieved on RBCs.

Detection of mistletoe lectins binding to tissues

The 3 lectins used in this study were designated as MLI, MLII and MLIII. Dectection and localization of binding of the 3 mistletoe lectins was achieved on tissues using antiserum to mistletoe lectins. The specificity of antisera raised for this study was confirmed by several techniques namely: ELISA, Ouchterlony and immunoblotting.

Albumin proved valuable in reducing non-specific staining and making interpretation easier.

Trypsin digestion has been reported to enhance staining of some lectins, such as HPA (Fenlon et al, 1987) and Con A (Walker, 1983).
In this study, trypsinization of breast cancer paraffin sections did not show any effect on subsequent binding of lectins, presumably reflecting the fixation used on these tissues as well as the configuration of the glycoproteins detected, being stable to fixation.

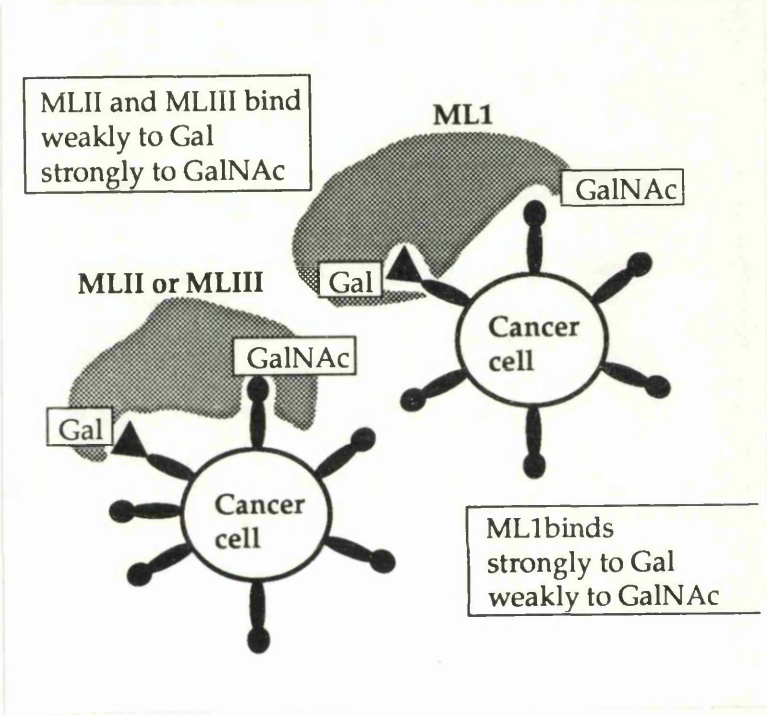
A total of 234 cases were stained for MLI, MLII and for MLIII. MLI binding was detected in 130 (55.5%) cases; MLII binding was detected in 135 (57.6%) cases; MLIII binding was detected in 132 (56.4%) cases.

Since no other reports on mistletoe lectin binding to breast cancer has been found in the literature, no comparison with other studies is possible at this stage. However, the expression of galactose and GalNAc on primary breast cancer cells has been demonstrated, using PNA (to galactose), SBA (to galactose and GalNAc), DBA (to GalNAc), and HPA (to GalNAc) (Franklin et al, 1983; Walker, 1984d; Walker ,1985; Leathem et al, 1983, 1985; Fenlon et al, 1986, 1987).

Heterogeneity of lectin binding to tissues

Out of the 234 cases stained for MLI, MLII & MLIII: 120 (51%) cases were +ve for the 3 lectins at the same time.
Heterogeneity in binding of lectins to different cancer cells was seen. the same population tended to bind with each of the 3 lectins, but varied in intensity of staining.
The possibility arises that this study is detecting 2 sugars on tumour cells. MLI binds strongly in the presence of galactose and weaker than MLII and MLIII in the presence of GalNAc. Conversely, MLII and MLIII bind strongly in the presence of GalNAc and weaker than MLI in the

presence of galactose. So cases where MLI showed binding stronger than that of MLII and MLIII perhaps are the cases where galactose is the predominant sugar on the tumour cell surface. While cases where MLII and MLIII showed a binding stronger than that of MLI, perhaps are cases where GalNAc is the predominant sugar. This possibility is illustrated in the following diagram:



However, some tumours failed to bind MLI although strong binding for MLII and for MLIII was detected. Conversely, a few cases were +ve for MLI while negative for MLII and MLIII. This heterogeneity perhaps can be explained by MLI having a major affinity to galactose and MLII & MLIII to GalNAc; perhaps these cases lack the expression of galactose and the expression of GalNAc is not in sufficient quantity for MLI to show binding , using the system in this study.

Although MLII and MLIII have a major affinity for the same sugar (Gal-NAc), in this study there were cases (only 3) that were positive for MLII but were negative for MLIII.

Heterogeneity using 2 lectins of the same simple sugar affinity but having different binding affinities has been reported by Walker (1984c),

who used 2 lectins, Lotus tetragonolobus lectin (LTA) and Ulex europeus I lectin (UEA), of the same sugar specificity, L-fucose, and found that the use of LTA alone could have suggested that in some breast carcinomas there is an absence of L-fucose-containing glycoproteins or glycolipids but this was be refuted by the demonstration of prominent reactivity with UEA in such tumours. Walker (1984) proposed that even when there is a lack of reactivity with both lectins in carcinomas it is probably better to confine statements to an absence or modification of the fucosyl oligosaccharides to which these lectins have a high affinity, rather than a total absence of L-fucose.

This study also illustrates the possible value of using two probes (MLII & MLIII) that show differences, even slight, in their inhibition by the same simple sugar, and shows that by their use subtle differences in the cell glycoconjugates can be distinguished. However, MLIII was shown to have a stronger affinity to GalNAc than that of MLII, so (for the same reason as described from MLI above) it is possible that the presence of GalNAc may be insufficient for detection by MLII using the system in this study.

In cases positive for the mistletoe lectins, not all the malignant cells showed binding. There were cells that were negative in some cases adjacent to cells that were strongly positive.

It is well recognized that primary malignant tumours are not composed of uniform cells with regard to metastatic behaviour, but contain subpopulations of cells with differing biological behaviour (Fidler and Hart, 1982; Liotta, 1988a). So it is possible that the binding of cells to mistletoe lectins reflect tumour cells in different stages of the cell cycle and with different subpopulations, that perhaps differ in their behaviour.

Usage of Iscador in Cancer therapy

An extract of mistletoe under the trade name of Iscador is widely used in the treatment of cancer, and reports of success in treating metastatic pleural effusions (Salzer, 1978), with increased in survival time (Hoffman, 1979, Leroi, 1979b) have been reported. An increase in the natural killer cells, Tumour necrosis factor (TNF), C-Reactive protein (CRP), interleukins and lymphokines in blood of patients with breast cancer after injection of Iscador has also been reported (Hajto, 1986; Hajto and Hostanska, 1989).

The anti-cancer activities of Iscador have been ascribed to a combination of cytotoxic and immunological reactions (Evans and Preece, 1973; Stirpe et al, 1980; Olsnes et al, 1982), although convincing evidence as to which component (s) is or are responsible for its reported action has not been produced.

However It has been suggested that perhaps the content of mistletoe lectin(s) may be of importance in Iscador activity (Hajto, 1986; Hajto et al, 1989).

To explore underlying reactions between mistletoe lectins and breast cancer, each of the 3 lectins was applied to breast cancer paraffin sections and the lectin binding to lymphocytes as well as to cancer cells was noted although the main results of staining (as positive or negative) were recorded on the basis of cancer cell reaction, ie, where in some cases lymphocytes stained positively while cancer cells were negative, these cases were considered negative.

In this study, some cases showed lymphocytes to stain positively while cancer cells were negative.

The A chain of MLI is known to act as a mitogen that effects a significant stimulation (Metzner et al, 1986). It would be conceivable that mistletoe lectins bound to the lymphocytes via their B-chain (carbohydrate-binding chain) by identifying receptors on lymphocytes surface and that the A chain will exert the mitogenic action.
In 1982, Barzilay et al, demonstrated mannose-specific membrane lectins on human lymphocytes. Mistletoe lectins (at least MLI) contain mannose (Luther et al, 1979) as has been shown by precipitation of the A chain as well as the B chain of MLI by Con A. Therefore, it is posible that the binding of mistletoe lectins to lymphocytes is the results mistletoe lectin uptake by mannose specific (Con A-like) lectins.
In these cases perhaps mistletoe lectins work on lymphocytes by binding to them and perhaps stimulating them, as mistletoe lectins were found to stimulate the production of lymphokines in patients blood and to increase many immunological parameter (Hajto, 1989).

The direct binding of mistletoe lectins to lymphocytes may suggest that in vivo perhaps MLs have some effect on lymphocytes. This may explain some pathway for immunomodulatory effects of Iscador. In cases where lymphocytes stained in addition to cancer cells, it is exciting to speculate that these perhaps were the cases that might receive the most benefit of Iscador treatment; both cytotoxic and immunomodulatory effect. In some cases lymphocytes were -ve while cancer cells were +ve. For these, the effect of Iscador perhaps might mediate directly on cancer cells rather than via immunological influence.

Obviously the cytotoxic and immunomodulatory effects seen with

mistletoe treatment needs further work to help understand the possible mechanisms of mistletoe lectin action and in particular Iscador therapy.

Prospective study

Not enough biopsies contained significant numbers of lymphocytes in this study, otherwise a correlation might have been sought to determine if binding to lymphocytes correlated with behaviour of these tumours. However, a study, in collaboration with Dr. Ann Clover at the London Royal Homeopathic Hospital, has started on patients with breast cancer who have been treated with Iscador. The purpose of this is to ascertain whether a prediction of response to treatment could be made by seeking binding of the patient's primary breast cancer tissues to the 3 mistletoe lectins. Then to correlate the results with reponse to Iscador treatment. This prospective study on Iscador patients has only just started.

Problems in predicting prognosis in breast cancer.

The biological behaviour of breast cancer is unpredictable. Of the many factors that have been reported to be of value in predicting prognosis and response to therapy ,i.e. **histology type and degree of differentiation** (Bloom and Richardson, 1957; Freedman et al, 1979; Davis et al, 1986); **primary tumour size** (Carlson and Stockdale, 1988); **hormone receptors** (Knight et al, 1977; Clark and McGuire, 1988); **oncogenes** (Slamon et al 1987; Barnes, 1989); **growth factors** (Sainsbury et al, 1985); and

proliferative rate and ploidy (Tubiana and Koscienly, 1988; McGuire et al, 1984; McDivitt et al, 1985), LN status emerges as the principal reliable method for defining groups with early recurrence (Fisher and Slack, 1970; Haybittle et al, 1982; Klintenberg et al, 1985; Benner et al, 1988; Saez et al, 1989). However, the trend to use more restricted surgical therapy for breast cancers reduces the amount of nodal tissue available for staging and has emphasized the need for an alternative marker for prognostic information from the primary tumour.

Problems related to the above factors are described briefly then discussed in detail in relation to this study:

Primary tumour size as a prognostic factor does not appear to be independent, but is related to the absence/presence of metastatic growth to the axillary LN(s) (Hartveit, 1989). It seems a weak prognostic factor in patients in whom the axillary LNs are not involved (Valagussa et al, 1978; Crowe et al, 1982).

Histological grading of the primary tumours involves great subjectivity and lack of reproducibility in assessment by different pathologists (Gilchrist et al, 1985). Stenkvist et al (1979) suggested that only between 60% and 77% of such grading results can be reproduced.

The most accurate assessment of prognosis might be by means of a **prognostic index** (Blamey et al, 1979), whereby several features are taken into consideration, but this again demands the sampling of LN(s) and takes in consideration the apical node involvement together with an acurate assessment of tumour grade.

The biochemical methods for assaying **steroid receptors** are still not

widely available. Monoclonal antibody estimation of hormone receptor status on simple histological specimens is currently being evaluated and may replace biochemical assays. However, these methods are very expensive.

The **amplification of the oncogene** c-erbB-2, emerges as a promising marker especially where it is associated with increased formation of the c-erb-B-2 protein, which detection possible on routinely-fixed sections (Berger et al, 1988), but it is only informative in a minority of women (10-30%) in whom the gene is amplified (Slamon et al, 1987; Wright et al, 1989; Barnes, 1989).

A new approach for predicting clinical behaviour of breast-cancer patients is the detection of carbohydrate expression in paraffin-embedded sections of the primary tumour:

Many studies have demonstrated carbohydrate expression in breast cancer sections (Franklin, 1983; Louis et al, 1983; Walker, 1984a, 1984b; Leathem et al, 1983; Leathem et al, 1984; Leathem et al, 1985; Stanley et al, 1986), and a range of lectins have been screened to identify carbohydrates in cancers with differing biological behaviour (Walker, 1984c, 1984d; Fenlon et al, 1986, 1987; Leathem and Brooks, 1987; Al-Alousi and Leathem, 1989c). None of these studies has examined binding of mistletoe lectins.

In this study the clinical progress of 234 patients, followed-up for up to 11 years, was related to staining of paraffin sections of their primary cancers by using 3 lectins from European mistletoe (Viscum album). The patients were not stratified by surgery, or treatment.

Mistletoe lectins binding: Survival and time to first recurrence

The patients studied.

Many cases in this study were lost to follow-up either immediately after operation (these cases were mostly private patients and overseas patients who came only for the operation) or sometime after the operation. Letters were written to the general practitioners of the patients for whom the address of their GPs was available, but unfortunately a poor response was obtained. However, this study still awaits more information on patients lost to follow-up from GPs and the follow-up to be updated. These cases were included in this study anyhow. The inclusion of these patients in the analysis was essential to reduce biasing of the study.

The cases that were lost to follow-up were included, their DFI and survivals were calculated as **one day after the date they were last seen.** The programe used was that of the Department of Radiotherapy and Oncology at the Middlesex Hospital, applying the Kaplan-Meier method (Friedman et al, 1983) for estimating the survival curve (the graphical presentation of the total survival experience during the period of observation) or lifetable (the tabular presentation). This survival analysis method is necessary in trials where subjects are entered over a period of time and have varying length of follow-up (as in this study). It permits the comparison of the entire survival experience during the follow-up. The advantage of using this method is the use of all patient follow-up information (although unequal) in estimating survival probabilities.

Survival correlated with staining .

In this study the rate of survival of all patients at 5 yrs was 74% and at 10 yrs was 60%.

At 5-yr interval, the rate of this study correpond very well with that of Wolff and Winzer (1989) and it is close to that of Polico et al (1988) (although slightly lower). It is higher than that reported by Sutherland and Mather (1988) and that reported by Karialainen et al (1989) for both Finnish & Estonian breast cancer patients (see following table).

Table

Survival (all patients)

Authors	5-yr	10-yr
Valagussa et al (1978)		60%
Haagensen (1986)		62%
Wolff and Winzer (1989)		73%
Karialainen et al (1989)	67% (Finnish population)	
	56% (Estonian population)	
Polico et al (1988)	78%	
Sutherland and Mather (1988)	57%	45%
Lunoetal(1988)	83%*	
This study	74%	60%

**patients' cancer was detected by mammography only, and by physical examination with and without an abnormal mammogram.*

As presented in the above table, at 10-yrs, the rate of survival of this

study corresponds extremely well with that of Valagussa et al (1978) and with that of Haagensen (1986). However it is much lower than that reported by Luno et al (1988) and much higher than that reported by Sutherland and Mather (1988).

Individual heterogeneity has an impact on survival analysis. The difference in psychology and attitude toward the disease in these different groups could affect the survival of the patients. Also the different centres reporting the survival rates perhaps have different management policy. In addition, here we are dealing with different populations and perhaps they differ in their survival owing to differences in their environment, diet and social and economical circumstances.

The results of staining of this study suggest that regardless of histopathological features, survival of patients with staining (stainers) for mistletoe lectins, especially MLIII, was shorter than those with no staining (non-stainers) at both 5 and 10 yrs intervals.

Table

Survival (all patients)

	5 yrs		10 yrs		*P-Value*
	S^1	Ns^2	S	Ns	
MLI	74%	80%	55%	76%	<0.0790
MLII	72%	80%	55%	80%	<0.0595
MLIII	71%	80%	54%	80%	<0.0441

1= Stainerts *2= Non-stainers*

In an attempt to identify an age group that might be strongly identified

with particular patients by the lectin staining in this study, patients were divided into 2 groups: those whose age was **equal or less than 50 yrs old** at time of diagnosis were grouped as one and those whose age was **over 50 yrs** were grouped as another group. There were not enough cases in this study, to divide them into smaller age groups (eg. 10 yrs difference between age groups). Those whose age equal and under 50 yrs were considered premenopausal and those over 50 yrs old were considered postmenopausal. Therefore, menopausal status in this study is a presumed status, owing to lack of information on menopausal status in the files of patients.

In this study, premenopausal stainers (whether for MLI, MLII or/and especially MLIII) were found to progress more rapidly than postmenopausal.

Table

Survival (In Premenopausal and Postmenopausal age groups)

		5 yrs		10 yrs		P-Value
		S	Ns	S	Ns	
MLI	Pre	60%	84%	47%	84%	<0.2708
	post	77%	81%	61%	74%	
MLII	Pre	56%	100%	43%	100%	<0.0950
	post	76%	76%	59%	76%	
MLIII	Pre	53%	100%	42%	100%	<0.0578
	Post	77%	75%	58%	75%	

This difference in prognosis between the 2 age groups was also found in relation to recurrence.

Recurrence correlated with staining

At 5-years, 55% of patients were disease free and 46% at 10-years. The rate at 5-yr of this study is lower than that reported by Polico et al (1988). At 10-yr the rate of this study is also lower than that reported by Fletcher et al (1989). However it is not far from that reported by Valagussa et al (1978) (as shown in the following table).

Table

DFI (all patients)

Authors	5-yr	10-yr
Valagussa et al (1978)		52%
Fletcher et al (1989)		55%
Polico et al (1988)	66%	
This study	55%	46%

The difference reported in the DFI rate over the period of 5- and 10-yr could be also explained as owing to differences in populations studied with their own individual heterogeneity (as explained for survival rates).

As with survival, stainers for mistletoe lectins seemed to do worse than non-stainers at both 5- and 10-yr intervals (shown in the following table):

Table
DFI (all patients)

	5 yrs		10 yrs		P-Value
	S	Ns	S	Ns	
MLI	52%	73%	36%	73%	<0.0031
MLII	47%	76%	35%	76%	<0.0013
MLIII	39%	77%	33%	77%	<0.0007

Although in this study the patients with different types of tumour and with metastases to different sites were grouped together, mistletoe lectins, especialy MLIII, seem to recognize marker(s) of aggressive behavior (high metastatic potential) in younger patients (as shown in the following tables).

Table
DFI (In Premenopausal and postmenopausal age groups)

		5 yrs		10 yrs		P-Value
		S	Ns	S	Ns	
MLI	Pre	38%	50%	22%	50%	<0.0036
	post	56%	83%	41%	83%	
MLII	Pre	33%	66%	21%	66%	<0.0022
	post	56%	78%	44%	78%	
MLIII	Pre	31%	71%	18%	71%	<0.0007
	Post	56%	78%	40%	78%	

The difference between the 2 groups of patients is age and perhaps the menopaual status i.e., patients with presumed different hormonal status. The relation of staining to hormonal status is discussed later.

The results of this study suggest that, regardless of histopathological features, LN status or primary tumour size, the metastatic potential of an individual breast cancer might be assessed, at least in premenopausal patients, by seeking binding of mistletoe lectins, especially MLIII, to primary tumour tissues.

Correlation of primary <u>size</u> with survival and DFI

The primary tumour size was divided into 3 groups <2 cm, 2-5 cm and >5 cm.

Strong correlation was found between tumour size and DFI ($p < 0.0009$) and with survival ($p < 0.00001$) The results are presented in the following table:

primary size/ DFI and survival

	5-year		10-year	
	DFI	*survival*	*DFI*	*survival*
<2cm	62%	88%	51%*	72%*
2-5cm	58%	82%	37%*	32%*
>5cm	0	20%	0	0 (5 cases only)

* *by extrapolation*

In this study, only 5 cases had tumours larger than 5cm , so proper correlation with the other tumour sizes (2-5cm and <2cm) was not possible. The presence of the >5cm group, which contains only 5 cases,

perhaps affected the statistics ($p < 0.0009$ for DFI and $p < 0.00001$ for survival) because these cases, although small in number, had such an awful outcome. However, differences in behaviour were seen between the groups of different primary size, indicating that the larger the tumour size the shorter the DFI and the survival. The inverse relationship between primary tumour size and DFI and survival has been reported before (eg. Hartviet, 1989).

Correlation between tumour grade and survival and DFI.

Some correlation was found between tumour grade and DFI ($p< 0.0631$) and with survival ($p<0.0334$). The results are presented in the following table:

Tumour grade/ DFI and survival

	5-year		10-year	
	DFI	survival	DFI	survival
grade III	35%	57%	29%*	37%*
grade II	66%*	100%*	66%*	100%*

* by extrapolation

Previous reporting starting from Bloom and Richardson (1956) indicated that the higher the tumour grade (the poorer the differentiation) the worse the prognosis (Freeman et al, 1979; Davis et al, 1986).

For grade 3 tumours in this study, survival rate at 5- yrs was 57% and at 10-yr was 37%. This corresponds well with Davis et al (1986) whose rate of survival for grade 3 tumours was 57% at 5-yrs, and with Freeman et al (1979), whose survival rate at 10-yrs was 33% for patients with grade 3

tumours. The rate in this study of being disease free at 5-yrs for patients with grade 3 tumours was 35%. This rate is lower but not far from that (42%) reported by Davis et al (1986).

No proper correlation could be done with grade I or grade II owing to the smaller number of patients analysed in this study.

Lymph Node (LN) status, Survival, and DFI.

Since histological evidence of axillary lymph-node (LN) involvement has so far been the most reliable marker in predicting patient's disease free interval (DFI) and therefore survival (Saez et al, 1989), this study also correlated LN involvement with survival and with DFI.

In this study, the number of LN involved were not taken into account. The LN status was entered as either LN+ve or LN-ve. The reason for this being that LN status in this study has been based on the pathologist's report. In some cases only one LN was sampled, if it was found to contain metastatic tumour the LN status of that patient was entered as positive. If that LN did not contain metastatic tumour then the LN status of that patient was entered as negative. Because of poor sampling it was considered more practical to record LN only as +ve or -ve.

Statistical analysis showed a strong association between LN status and survival ($p < 0.0006$).

Survival

At 5 yrs interval, the rate of survival of this study (89% for LN-ve and 67% for LN+ve) correlated very well with that reported by Haagensen

(1986) as outlined in the following table:

	5-year survival	
	Haagensen (1986)	This study
LN-ve	87%	89%
LN+ve	67%	67%

At 10 yrs, the rates of survival of this study in relation to LN status were correlated to survival rates reported by the authors in the following tables:

	10-year survival				
	Haagensen[1]	Fisher[2]	Valagussa[3]	Cutler[4]	This study
LN-ve	74%	65%	82%	75%	84%
LN+ve	51%	25%	40%	30%	47%

1 = Haagensen (1986) 2 = Fisher et al (1975)
3 = Valagussa et al (1979) 4 = Cutler et al (1969)

The LN-ve rate of survival in this study corresponds well with that reported by Valagussa (Valagussa et al, 1978), higher than that reported by Haagensen (1986) and by Cutler et al (1969), and much higher than that reported by Fisher (Fisher et al, 1975).

These differences perhaps might be explained by differences in the groups studied.

In this study, statistical analysis showed a strong association between LN status and DFI ($p < 0.0001$).

Recurrence or Disease Free Interval (DFI).

At 5-years interval, the cumulative proportion disease free was 75% for patients without LN involvement and 36% for those with LN involvement lower than that reported by Klintenberg et al (1985).

	5-year DFI	
	Klintenberg et al (1985)	This study
LN-ve	83%	75%
LN+ve	57%	36%

At 10 yrs interval, the results of this study corresponds extremely well with rates reported by Fisher et al (1975)[1] and Valagussa et al (1978)[2] (as outlined in the following table). However, the rate for LN+ve patients is much lower than that of Fletcher et al (1989)3.

	10-year DFI			
	Fisher1	Valagussa2	Fletcher3	This study
LN-ve	75%	75%		71%
LN+ve	25%	28%	44%	27%

Correlation of Primary size, grade, LN status and ML staining with survival and DFI:

	Survival			DFI		
	5-yr	10-yr	P-value	5-yr	10-yr	P-value
<2cm	88%	72%		62%	51%	
2-5cm	82%	52%	< 0.00001	58%	37%	< 0.0090
>5cm	20%	0		0	0	
Grade II	100	100⊕		66	66*	
Grade III	57%	37%	< 0.0613	35%	29%	< 0.0334
LN+ve	67%	47%		36	27	
			< 0.0006			< 0.0001
LN-ve	89%	84		75	72	
MLI/S	74%	55%		52%	36%	
			< 0.0790			< 0.0031
MLI/NS	80%	76%		73%	73%	
MLII/S	72%	55%		47%	35%	
			< 0.0595			< 0.0013
MLII/NS	80%	80%		76%	76%	
MLIII/S	71%	54%		39%	33%	
			< 0.0441			< 0.0007
MLIII/NS	80%	80%		77%	77%	

⊕ = 24 patients but 6 lost to follow-up.

* By extrapolation

Based on the p-values (values to see if the observed differences between the different groups could be just chance (Peto et al, 1977) , **For the survival analysis, it appeared that primary tumour size is the best predictor for tumours 2-5cm, followed by LN status, MLIII, MLII, by tumour grade then by MLI.**

The value of tumour size has been reported to be dependent on LN status (Haagensen, 1986; Hartveit, 1989). On its own, tumour size is a weak prognostic factor in patients with LN-ve (Crow et al, 1982). In this study, with tumour grade analysis a proper correlation between the 3 grades was not possible as the number of patients with grade I and grade II was low. This is not surprising, as generally breast cancers retain little evidence of differentiation (Carlson and Stockdale, 1988).

This study confirms many reports (Fisher et al, 1975; Valagussa, 1978; Fisher et al, 1983) indicating that LN status as a strong predictor of survival.

Based on the P-values for DFI analysis, **LN status again appears the best predictor, followed by the mistletoe lectins, then by tumour size and lastly tumour grade.**

LN status as a predictor of recurrence ($p < 0.0001$) was superior to MLIII binding, which was the most significant ($p < 0.0007$) of the 3 mistletoe lectins examined. However, the need to find an alternative marker for prognostic information, using primary tumour tissue, is greater than ever owing to the trend toward more conservative surgery. Mistletoe lectins, especially MLIII, perhaps could represent an alternative: they appeared able to predict metastasis at least in premenopausal patients ($p < 0.0007$); their application to formalin-fixed sections is

simple and if mistletoe lectins prove to be applicable to fine needle aspirations (FNAs), then the application of these lectins might have the advantage of speed and produce valuable staging information to surgeons in outpatients clinics. However, a previous experience (Al-Alousi and Leathem, 1989D) using HPA on FNAs showed that while HPA binding is demonstrable in cytological smears, the problem was the availability of good and adequate aspirations. Perhaps with such good and reliable aspirations, useful markers could be applied and provide a rapid staging service to the surgeons and patients in out-patient's clinic.

Metastasis

Primary breast cancer does not usually pose an immediate threat to the life of patients. Rather, it is the probability of metastatic spread and recurrence which determines the survival of the breast cancer patient (Furmanski et al 1981).

By the time of diagnosis, human neoplasms can contain multiple populations of cells with differences in such properties as growth rate, cell-surface receptors and products, response to cytotoxic agents, invasion and metastatic potential (Fidler, 1989).
Several studies, beginning with Paget's (1889) classic observation on **"seed and soil"** sought to understand the relationship between the primary cancer in breast and its spread (Fidler, 1976; Weiss, 1988; Sugarbaker, 1981; Tarin and Price, 1981; Nicolson, 1988a, 1988b, 1989a, 1989b; and Liotta, 1989a, 1989b).

Tumour metastasis is a complex series of sequential steps involving the

growth and invasion of malignant cells at primary sites, their penetration into lymphatic, blood circulation and body cavities, transport to distant sites, implantation at these sites, and invasion and growth (Nicolson, 1988a , 1988b; Nicolson, 1989a, 1989b; Liotta, 1989a, 1989b; Narita, 1989).

A variety of tumour cell adhesive interactions are important in blood-borne metastasis (Nicolson 1982, 1983; Belloni et al, 1986). To spread to distant sites malignant cells must enter the blood and be released into the circulation. The adhesion of circulating malignant cells to one another, to circulating normal cells, such as lymphocytes and platelets and to specific microvessel endothelial cells and their underlying subendothelial matrix basement are important events in determining organ-specific metastasis (Nicolson 1988a, 1988b, 1989a, 1989b; Feldman and Eisenbach, 1988; Liotta, 1989a, 1989b). Once adhesion to the endothelium occurs, malignant cells can stimulate endothelial cell retraction, exposing the underlying basement membrane and ultimately invading this structure.

Cell surface glycoconjugates and lectins in metastasis:

A variety of data implicate a role for cell surface glycoconjugates and for endogenous lectins {carbohydrate receptors that could be demonstrated by using antibodies against the native lectin (Mermomsky et al, 1986)} in important steps of the metastatic process. Specific changes in glycoconjugates on malignant cells are closely associated with metastasis formation in some metastatic systems, and tumour cell metastatic- and endothelial cell-binding properties can be affected by metabolic alterations in tumour cell surface oligosacchariudes (Nicolson, 1988a, 1988b; Dennis et al, 1982; Dennis and Laferte, 1989).

Data have strongly supported a role for the cell surface in the ability of tumour cells to detach and metastasize (Nicolson, 1984, 1988a, 1988b; feldman and Eisenbach, 1988). In particular, many studies have shown direct correlations between the metastatic phenotype and altered glycosylation of cell surface glycoconjugates (Dennis and Kerbel, 1981; Dennis and Laferte, 1989). For example, alterations in cell surface sialic acid have been implicated in changing the adhesive capabilities of tumour cells (Dennis et al, 1982).

Lectins, because of their specific carbohydrate-binding properties, have been used extensively as probes to study the surface architecture of normal and transformed cells (Newman et al , 1979; Tao and Burger, 1977; Leathem et al, 1984; Leathem, 1986; Kimber, 1988).

Endogenous tissue lectins:

Lectin-like molecules have been detected on the cell surface of several human and murine tumour cell lines (Raz and Lotan, 1981; Gabius, 1987). Interestingly, normal tissues are reported to contain lectins which can mediate binding to tumour cell surface glycoconjugates (Schlepper-Schafer et al, 1981).

Breast cancer glycoconjugates and metastasis:

In breast cancer, several reports suggest that lack of reactivity of breast cancer cells with certain lectins, such as Pokeweed lectin (Al-Alousi and Leathem, 1989c) and Wheat Germ Agglutinin WGA (Walker, 1984b), is related to the potential of cells to metastasize to local LN. There are also reports suggesting that binding of malignant breast cells to certain lectins is related to the metastatic potential of those cells, such as with Con A (Walker, 1984) and Helix pomatia agglutinin, HPA (Leathem et al, 1985; Leathem and Brooks, 1987, Fenlon et al, 1987).

HPA, like MLII and MLIII, is a lectin that has an affinity to GalNAc. The expression of GalNAc as detected by HPA was proposed to be a marker of breast cancer metastasis (Leathem et al, 1985, 1987). This was found especially in premenopausal women. The results of the study described here correspond well with that of Fenlon et al (1987), in that GalNac binding lectins appear to be related to disease progression and with that of Leathem and Brooks (1987) in that the expression of GalNAc, in this study as detected by MLII and MLII, is associated with rapid metastasis, especially in premenopausal women.

Perhaps HPA and the MLII and MLIII are recognizing the same receptors in tissues but this is speculation until the receptors are isolated and characterized. Certainly, a further study to identify and characterize the receptors of these lectins might contribute to the understanding of biological behaviour and metastasis of breast cancer.

Lectins, glycoconjugates and Hormone receptors:

Are the structures stained by MLI, MLII and MLIII in postmenopausal patients hormone dependent?

In the rat mammary gland, PNA-binding sites were reported to be hormone dependent (Newman et al, 1979).

Klein et al (1981) have shown a correlation between HPA and PNA binding on human breast carcinoma tissues and ER status; positive staining indicating sites of hormone dependent cell secretion. This was confirmed by Walker et al (1985), for PNA, who reported that the reactivity of breast carcinoma to PNA is related to the presence of oestrogen, but not pregesterone receptor. Walker et al (1985) also found a relationship between breast carcinoma staining for SBA and the presence/absence of oestrogen, but not of progesterone, receptor.

The findings of Klein et al and Walker et al were not confirmed by Stanely et al (1986), who showed that PNA-binding and ER status are two independent factors, and by Fenlon et al (1987), who did not show a correlation between HPA-binding and ER status.

This study adds further evidence for a role played by carbohydrate expression in metastasis of cancer cells (Nicolson, 1988a, 1988b; Dennis and Laferte, 1989) and therefore in deciding the progress of the disease.

Breast cancer cause so much despair and frustration to women, 15 - 20% of whom are under the age of 45 (Parente et al, 1988). Every 13 minutes one women is diagnosed as having breast cancer (Parente et al, 1988) and this disease appears to be increasing in incidence and remains hard to cure. The work shown in this thesis is an attempt to try to find a way to help women with breast cancer to fight a disease against which, at present, they rarely win.

The value of this work

Mistletoe lectins are inexpensive tools and investigations have shown that the mistletoes throughout the world are rich sources of lectins.

Mistletoe lectins have been shown to be able to predict the metastatic potential independently of tumour grade, primary tumour size and LN status, at least in premenopausal patients by a simple application to routinely fixed paraffin-embedded sections of primary breast cancer. However, such a test would need further evaluation on breast cancers from other centres both retrospectively and prospectively.
In addition since the mistletoe lectins are toxic their use as routine laboratory agents demands some care and precautions.

CONCLUSION

Three lectins were isolated from the European mistletoe plant (*Viscum album*), designated as MLI, MLII and MLIII, with major sugar specificities for galactose (MLI) and N-Acetyl-Galactosamine or Gal-NAc (MLII and MLIII).

In formalin-fixed paraffin-embedded sections of 234 cases of primary breast cancers, Mistletoe lectins, especially MLIII, appeared to recognise carbohydrates that are associated with increased metastatic potential especially in premenopausal patients.

WHAT TO DO NEXT

This retrospective study has opened the way to several ideas and projects. The following future projects would seem to be important:

1- A prospective study to make sure that the binding and results claimed in the retrospective cases (paraffin sections) were not just an artifact. This study is already underway, using tissues from patients who have been treated with mistletoe injections (Iscador) for their breast cancer.

2- Find out if the binding of mistletoe lectins could be demonstrated using fine needle aspiration (FNA) cytology.
This could be achieved by seeking binding of FNAs to mistletoe lectins and correlate this with the lectin binding of corresponding paraffin-embedded sections of the excised tumour from patients who are carefully followed-up.

3- Further study to identify and characterize the receptors of mistletoe lectins, especially of MLIII, might contribute to the understanding of biological behaviour and metastasis of breast cancer. The isolation of the material that mistletoe lectins bind to might be achieved by:
   i- Affinity chromatography: by using beads coated with mistletoe lectin(s) and passing a homogenate of fresh primary tumour that was shown to have binding sites to the lectin, down a column.

The bound material could be then eluted and analysed further.

ii- Other isolation procedures before affinity chromatography, ie, ion-exchange chromatography or gel exclusion chromatography.

iii- Testing the eluted material by PAGE in order to ascertain its purity and its molecular weight.

4- To study retrospectively other hormone-dependent tumours, such as ovarian tumours, to see whether mistletoe lectins might also recognise those with a bad prognosis. This could be achieved by staining formalin-fixed paraffin-embedded sections of the primary tumours with mistletoe lectins and then correlating the binding with clinical behaviour of these patients.

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APPENDICES

APPENDIX (1)

(Phosphate buffered saline "PBS")

Ready made tablets of PBS from Oxoid Ltd, were made to PBS solution of pH of approximately 7.3, according to the manufacture instructions.

APPENDIX (2)

Preparation of saturated ammonium sulphate

760g of solid ammonium sulphate was made up to 1 litre with DW. The mixture was put on stirrer + heater to dissolve the ammonium sulphate and to make a "supersaturated solution". At room temperature the excess ammonium sulphate precipitated, leaving a saturated solution (Heide and Schwick, 1973).

APPENDIX (3)

Determining the best ratio of crude mistletoe extract to the ammonium sulphate for precipitating lectins

The salt concentration at which each protein precipitates is different, but between closely related molecules the difference is not sufficiently great to give high-grade purity of the precipitate. However, it is useful as a first step in isolation as many unwanted molecules will remain in solution.

Ten tubes were prepared:

Tube 1 contained 9 mls of extract + 1 ml saturated (NH4)2SO4
Tube 2 contained 8 mls of extract + 2 mls saturated(NH4)2SO4
Tube 3 contained 7 mls of extract + 3 mls saturated(NH4)2SO4
Tube 4 contained 6 mls of extract + 4 mls saturated(NH4)2SO4
Tube 5 contained 5 mls of extract + 5 mls saturated(NH4)2SO4
Tube 6 contained 4 mls of extract + 6 mls saturated(NH4)2SO4
Tube 7 contained 3 mls of extract + 7 mls saturated(NH4)2SO4
Tube 8 contained 2 mls of extract + 8 mls saturated(NH4)2SO4
Tube 9 contained 1 ml of extract + 9 mls saturated(NH4)2SO4
Tube 10 contained the crude extract only used as positive control.

The mixtures in the tubes were allowed to settle overnight at 4°C. The precipitate was collected by centrifugation at 10,000 for 10 minutes, dialysed overnight against DW and then resuspended in a minimum volume of 0.5 M Nacl. The precipitated proteins of each tube were subjected to haemagglutination test (as described in section 2.1.1.2.) to find out which tube contain the highest concentration of the lectin. Tube No. 3 (7 parts extract : 3 parts ammonium sulphate) was found to contain the highest concentration.

APPENDIX 4

GEL CHROMATOGRAPHY

The mechanism of action of gel chromatography is:

1- Particles of a gel matrix (with pores of controlled dimension) are poured into a column under conditions which permit uniform packing of the particles, thus forming the bed.

2- The sample, consisting of a mixture of substances which differ in

molecular dimensionsis then applied to the bed surface and subsequently caused to perculate through the bed by eluent flow.

3- As elution proceeds, molecules which are too large to enter the pores of the gel matrix pass rapidly through the bed in the space surrounding the gel particles, and are eluted in a single zone near the begining of the elution profile, while molecules which are capable of diffusing into the pores of the matrix are retarded in their migration through the bed. The extent of retardation is inversely correlated to molecular dimensions; therefore, the smallest molecules are retarded to the greatest extent and are thus the last to emerge from the bed. Elution of all sample solutes is usually complete when a volume of eluent approximately equal to the total bed volume has passed through the bed.

Material and equipments

Bio-Gel P-200 (Bio-Rad)
Tris Buffered Saline (TBS)
A chromatography column (Pharmacia)
A Peristaltic pump
A fraction collector
Sample (clarified Mistletoe sample)

Method

1- 15 g (dry weight) of Bio-gel was left in 500 mls TBS at $4^{o}C$ overnight. This provided sufficient swollen gel for a 80 X 2.6cm column (each gram of dry gel makes a bed of 25mls).

2- The gel was de-gased under a vacuum to remove air bubbles.

3- The gel was then poured into the column along a glass rod to avoid air bubbles. The gel was all poured without stopping. the outlet of the

column was left opened during packing.

4- Once the gel has settled, the flow was started and the column was equilibirated by running through at least two column volumes of buffer (TBS). Then the flow rate was adjusted using the peristaltic pump.

5- When the all buffer drained from the column without letting the top of the column get dry, the outlet of the column was closed. After ensuring that the bed surface was even, a nylon net was placed gently on it and the sample (10 mls) was layered gently onto the gel surface using a long Pasteur pipette. A little sucrose was added to the sample before application in order to increase its density and help even loading.

6- The outlet of the column was opened and the flow started at a flow rate of ml/hr using a peristaltic pump.

7- The fractions were collected using a fraction collector.

APPENDIX (5)

Tris buffered saline "TBS"

Concentrated (X10) TBS was made by dissolving 600.57g Tris (Sigma)+ 850g Nacl (Sodium chloride) in 1 10 litres distilled water. The pH was adjusted to 7.6 with concentrated HCl. Each litre was made up to 10 litre with distilled water when used.

APPENDIX (6)

ION-EXCHANGE CHROMATOGRAPHY

Material and equipment

DEAE -DE52 (Whatman)

100 mls 0.5 M Nacl

Column and fraction collection apparatus

Linear Gradient device

Sample

Method

1- The pre-swollen ion-exchanger (DE-52) was placed in a beaker (5g was used for every 1 ml sample) containing cold distilled water (DW). The resin was allowed to settle.

2- The supernatant which contained "fines"; (which may block the column), was removed. Washing with DW and discarding the supernatant was repeated 5 times.

3- The slurry was poured into the column with the outlet of the column open.

4- The column was packed by pumping DW through at least twice the volume of the column.

5- When the elute reading of the spectrophotometer (absorption at 280nm), which has been blocked against DW gave a zero reading, the sample was applied to the column.

6- The sample, which has been dialysed against DW, was applied to the column and pumped through using DW. The effluent was

monitored for protein presence using spectrophotometer (absorption at 280nm).

7- The bound proteins were eluted with a 200 mls Nacl molarity gradient.

8- Two mls fractions were collected using a fraction collector.

APPENDIX (7)

Glycine-Hcl buffer, pH 2.6 (Hudson and Hay, 1977).

500 mls of 0.2 M glycin (glycin in aminoacetic acid) was prepared, adjusted to pH 2.6 with 0.2N Hcl, and made up to 1 litre with distilled water.

APPENDIX (8)

Poly Acrylamide Gel Electrophoresis (PAGE)

8.A. Table showing different molecular weight ranges using different concentrations of the polyacrylamide gel

Table ; 1

Percentage acrylamide	*15%*	*12%*	*10%*	*7.5%*
Molecular weight range	*8,000-50.000*	*10,000-75,000*	*20.000-100.000*	*40.000-150.000*

8.B. Principle behind the stacking gel effect

The band sharpening effect of the stacking gel, relies on the fact that negatively charged glycinate ions have a lower electrophoretic mobility than the protein-SDS complexes, which in turn have a lower mobility than the Cl- ions of the loading buffers and stacking gel. When the current is turned on, all the ionic species have to migrate at the same speed otherwise there is a break in the electric circuit. The glycinate ions can only move at the same speed as the Cl- if they are in a region of high electric field. Field strength is inverted proportion to conductivity, which is proportion to concentration. The 3 species (glycin, protein-SDS, cl-) adjust their concentrationm so that Cl-> protein-SDS > glycinate. There is only a small quantity of protein-SDS , therefore it tightens into a band between glycin and Cl- boundries. Once the glycinate reaches the running gel it becomes more fully ionized in the high pH and its mobility increases, leaves behind the protein-SDS to electrophores at their own rates.

8.C. Materials and reagents for PAGE

Materials

A vertical dual mini slab gel system (Atto Corporatio)

Power supply (Bio-Rad).

Reagents:

Solution A

30% acrylamide solution (29.2g acrylamide + 0.8g N,N-methylenebisacrylamide "bis" + distilled water to make up to 100ml).

Solution B

Gel buffer "1.5M Tris buffer, pH 8.8" (18.17g of tris and 0.4g of SDS

dissolved in water, adjusted to pH 8.8 with HCl and made up to 100 ml with distilled water).

Solution C

Stacking gel buffer "0.5M Tris buffer, pH 6.8" (6.06g of tris and 0.4g of SDS dissolved in water, adjusted to pH 6.8 with HCl, and made up to 100mls with distilled water).

Solution D

10% ammonium persulphate (25mg ammonium persulphate in 250ul distilled water, usually prepared fresh just before use).

Solution E

N,N,N'N'-tetramethylethylene diamine (TEMED)

Solution F

Aqueous butanol

8.D. PAGE/ Method

Solution A was degased and mixed with solution B (or Solution C) and water (for ratios see Table; 2). Solution E and solution D were added just before casting the gel. The mixture was gently mixed and the gel(s) was immediately applied between the 2 plated provided for running the gel. the plates were filled up to 4cm from the top of the plates. The top of the gel was overlayed with solution F, aqueous butanol water, (Water saturated with distilled water). This ensures that the polymerized gel will have a flat surface. The gel was left then to polymerize for 30-60 minutes. The butanol water was washed off with 3 changes of distilled water and the surface of the gel was dried

with filter paper. The stacking gel was mixed and dispensed over the separating gel. Immediately the plastic comb for forming the sample wells was inserted and was left to polymerize for 30 minutes. Before use, the comb was gently removed and wells were filled with running buffer before application of the samples.

Table; 2

Composition: (quantity; in ml) (%; gel concentration)

	Separation gel			stacking gel
	7.5%	10%	12.5%	5%
Solution A	4.5	6	7.5	0.9
Solution B	4.5	4.5	4.5	
Solution C	-	-	-	1.5
Solution D	0.07	0.07	0.07	0.07
Solution E	0.01	0.01	0.01	0.01
Distilled Water	9	7.5	6	3.6

8.E. Sample preparation for PAGE

Protein concentration in sample: 1-2mg/ml.

1% SDS

1% 2-mercaptoethanol

10mM Tris buffer, pH 6.8

20% Glycerine

Bromophenol blue (a stained marker)

The samples were heated for 1 to 2 minutes at 100^{o}C before application.

8.F. Molecular weight (MW) standards for PAGE

The markers used were obtained from sigma ready to use.
The SDS-PAGE prestained markers were of a MW ranged from 25K-180,000K. They were:

MW	Protein
180,000	Alpha 2 Macroglobulin (human palsma)
116,000	B galactosidase (E. Coli)
84,000	Fructose-6-phosphate kinase (rabbit muscle)
58,000	Pyruvate kinase (chicken muscle)
48,000	Fumarose (pig heart)
36,000	Lactate dehydrogenase (rabbit muscle)
26,000	Triosephosphate Isomerase (rabbit muscle)

8.G. Running the polyacrylamide-gel

1- The gel apparatus was set, the buffer tanks were filled with running buffer containing SDS.
2- The samples were added to the wells with a Hamilton syringe.
3- The power supply was then connected and the gel was run at constant current supply: 160 V until the bromophenol blue marker was about 1cm from the bottom of the gel.
4- The gel then was ready for staining and molecular weight estimation

8.H. Coomassie blue Staining of polyacrylamide-gel

i) Staining

Distilled water was added to 2.5g of Coomassie Brilliant blue, 500ml of methanol and 100mls of acetic acid, and was made up to 1000ml. The gel was left in this staining solution for a minimum period of 5 hrs with gentle shaking.

ii) Destaining and storing

Distilled water was added to 250 ml of methanol and 70 ml of acetic acid, and made up to 1000 mls. The stained gel was left in this destaining solution for a minimum period of 12 hrs with many changes of the solution and gentle shaking. The gel then was rinsed well with 10% methanol, 10% acetic acid and stored in 7% acetic acid.

APPENDIX (9)

Ouchterlony gel double diffusion

Plates were prepared using 1% agarose in phosphate buffered saline (PBS) (0.02 M sodium phosphate containing 0.154 M Nacl) pH 7.2. The gel was left to set, then wells were made using a devised instrument that allow a degree of accuracy in both the cutting of the wells and in their placing. The wells were filled with 20 µl of samples (in 1% PBS).

APPENDIX (10)

Enzyme-linked Immunosorbent Assay (ELISA)

- Tenfold dilutions (10-1-10-4) of the 3 different mistletoe lectins in carbonate buffer*pH 9.6 (starting dilution was made by mixing 20 µl lectin + 180 µl buffer) were made in the ELISA plate itself and incubated overnight at room temperature (RT) in a damp box.
- The mixture was then discarded and the wells of the plate were then washed with PBS/tween**, pH 7.4 3 X 3 minutes
- 200µl of rabbit anti-ML was added to each well. As negative control, Ig fraction of normal rabbit serum was diluted in PBS/tween and used. the plate was incubated for 2 hrs at RT.
- The mixture was then discarded and the wells of the plate were then washed with PBS/tween 3 X 3 minutes
- 200µl of peroxidase labelled swine anti-rabbit Ig in dilution of 1: 400 in PBS/tween was added to each well and incubated for 2 hrs at RT.
- The mixture was then discarded and the wells of the plate were then washed with PBS/tween 3 X 3 minutes
- 200 µl of Diaminobenzidine (DAB)/Hydrogen peroxide (H_2O_2) (Appendix: 12) was added to each well and incubated for 30 minutes then results were read. Positive reactions gives degree of brown colour depending on the intensity of reaction. Negative results or no reaction gives colourless end product.

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* Carbonate buffer pH 9.6: 1.59g of Sodium carbonate (Na2CO3) + 2.93g of Sodium hydrogen carbonate (Na2HCO3) were dissolved in 1 litre distilled water. The pH was checked before use.

** PBS/Tween pH 7.4: To each 100mls PBS, 0.5 ml of detergent Tween 20 was added.

APPENDIX (11)

Immuno-dot blotting

A. Nitrocellulose Membrane Preparation

With a pencil, a rectangular grid was drawn onto NCM (Anderman). The membrane was then washed with distilled water for 5 minutes and left at room temperature to dry. The NCM was handled throughout with forceps.

B. Dotting

When the membrane was dry, a small drop (20 µl) of MLI, MLII and MLIII was placed into each square using a Hamilton syringe. Four strips of NCM containing the 3 lectins in each were prepared and these were left to dry thoroughly.

C. Immunoperoxidase staining (Nakane and Pierce, 1966) (Diagram is shown below)

i) Blocking

The NCMs were placed in a staining tray and were treated with 3% bovine serum albumin in phosphate buffered saline (PBS) containing 0.05% detergent (tween 80). The tray was then left on a gentle shaking for a minimum period of 30 minutes. This treatment results in the blocking of non-specific antibody binding sites on the membranes.

ii) Incubation with the test antibody

After decanting the blocking solution, The polyclonal anti-mistletoe Ig which has been labelled to peroxidase (POD) in East Berlin/ Staaltich Institut fur Nahrmedian and immunpraparat, was added to each strip except one which was used as negative control. Different dilutions of the Ig were used. To one strip 1:100 of POD/Anti_MLI Ig was added. To the second strip 1:200 POD/Anti-MLI Ig was added. To the third strip 1:400 POD/Anti-MLI Ig was added. The 4th strip was left in

PBS/tween and was used as negative control. The strips were incubated with the antibody for 2 hrs.

iii) Washing and development

After removing all the unbound solutions, the NCMs were washed thoroughly with 3 changes of PBS/tween over a period of 45 minutes. After removing the washing solutions, DAB/H_2O_2 (appendix: 12) was added in order to locate the antibody binding labelled with peroxidase. The NCMs were left in the developing solution for 10 minutes with gentle shaking. Positive reaction was seen as a brown dot against a white background.

iiii) Washing and storing

The developing solution was poured off and the NCMs were washed thoroughly with distilled water. The NCMs were allowed to dry between two sheets of filter paper. Once dry, the NCMs were kept in the dark as the colour of reaction might fade if left in the light.

APPENDIX (12)

Diaminobenzidine/H_2O_2

This mixture was prepared as a 1g of Diaminobenzidine (DAB) (Sigma) in 200 mls distilled water stock solution, which was aliquoted into 1ml aliquotes and stored at - 20°C until use. Just before use one aliquot was diluted to 10 mls with TBS and 60ul of H_2O_2 (30% aqueous solution) was added.

APPENDIX (13)

Western blotting

- The graphite plates of the semi-dry blotter (ancos) were washed with distilled water.

- Six layers of filter paper (Whatman No. 1) which had been soaked in Anode buffer I (0.3M Tris, 20% methanol) were placed on the anodic graphite plate, making sure that no air bubbles were caught within the filter papers. Air bubbles create points of high resistance resulting in "bald" spots on the membrane.

- On top of the 6 layers of filters, 3 layers of filter paper which were soaked in Anode buffer 2 (25 mM Tris, 20% methanol) were laid making sure that no air bubbles were traped within the filter papers by rolling with a cylinder or a bottle in one direction. This was followed by NCM which had been briefly rinsed in distilled water.

- The SDS-PAGE gels to be blotted were then placed on top of the NCM and care was taken to remove all air bubbles. A further 9 layers of filter paper this time soaked in Cathode buffer (25 mM Tris 40 mM 6-amino-n-hexanoic acid, 205 methanol), were placed on top of the SDS-PAGE gel.

- Finally, the essembly was covered with the apparatus lid to which the cathode plate is attached .The gels were thus firmly and evenly pressed against the NCM. The apparatus was then connected to a power supply with a constant 0.8 mA/cm2 for 1hr at room temperature.

- After that the current was disconnected and the blotted NCM was

collected. To check that the proteins have been transferred, before starting any immunological staining, the NCM was stained for few minutes with Ponceau red stain. The NMC was then washed with distilled water to visualize the bands of the transferred proteins which appeared red against the white background. If washing was continued with distilled water, all the staining eventually disappeared.

- Once the transfer of the protein was confirmed, the immunoperoxidase staining was performed. The staining method used was same as described above (in dot- immunbinding assay, appendix 11) using the optimal dilution of peroxidase conjugated anti-MLI Ig (1:200 in PBS/tween).

Appendix 14

Lectin-peroxidase staining method

1- The formalin-fixed paraffin-embedded sections of primary breast cancer were de-waxed in xylene for 10 minutes.

2- The sections were taken from xylene into 100% alcohol, to 95% alcohol then to running tap water and left to be washed for 5 minutes.

3- The sections then were put in a bath of hydrogen peroxide (H_2O_2)/Methanol (40 mls of 30% vol. H_2O_2 + 400 mls methanol) and left for 20 minutes.

4- The sections were washed for 5 minutes under running tap water then washed briefly with Tris buffered saline (TBS) pH 7.2.

5- The lectins then were applied to the sections in different dilutions: MLI: 200 µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, & 6µg/ml.

MLII: 200 µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, & 6µg/ml.

MLIII: 200 µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, & 6µg/ml.

TBS was used to dilute the lectins . The sections were incubated with the lectins for 2 hrs.

6- The section then were washed with 2 changes of TBS 10 minutes each wash.

7- Then peroxidase (POD) conjugated polyclonal MLI (POD/Anti-ML) antibody was applied in different dilutions to each dilution of the lectin: 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6µg/ml & 3µg/ml. The antibody was diluted with TBS. Incubated for 1 hr.

8- Washing as in step No 6 was repeated.

9- The binding was visualized using Diaminobenzidine + H_2O_2. The DAB (Sigma) was prepared as described in appendix (7). The sections were then left in the developing solution for 10 minutes .

10- Sections washed under running tap water for 5 minutes.

11- Sections counterstained with Mayer haematoxylin for 3 minutes then washed under running tap water for 5 minutes to blue.

12- Sections were dehydrated through alcohol to xylene and then mounted (using Depex).